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14. ABSTRACT: There is an epidemic of obesity in the military. Obesity leads to insulin resistance syndromes, notably metabolic syndrome and type 2 diabetes. The major cause of death in these syndromes is atherothrombotic vascular disease, including coronary artery disease. Therefore, when retired military personnel and their families reach middle age, there will be an epidemic of obesity-related vascular disease. We have made major progress on the key Tasks over the last year. We have completed our study on a commonly used drug for obesity-related diabetes, pioglitazone, showing that it promotes plaque progression in vivo (published in Circulation). This study is very important, because recent clinical studies have implicated this class of drugs in heart disease. Indeed, pioglitazone is being used and studied in military personnel. We have also made major progress in understanding how a particular property of adiponectin, a "beneficial" hormone that is decreased in obesity, may protect against obesity-related heart disease. Finally, we have made key discoveries related to apoptosis signaling that are relevant to the atherogenic action of angiotensin-2, a hormone that is increased in obesity. These studies set the stage in a completely new way for studying how this hormone promotes atherosclerosis in obese subjects.

15. SUBJECT TERMS
obesity, diabetes, insulin resistance, heart disease, atherosclerosis, macrophages, apoptosis, unfolded protein response, PPAR, adiponectin, angiotensin 2

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INTRODUCTION:

As described in detail in the original grant application, there is an epidemic of obesity in the military. Obesity leads to insulin resistance syndromes, notably metabolic syndrome and type 2 diabetes. The major cause of death in people with insulin resistance syndromes is atherothrombotic vascular disease, including acute myocardial infarction, sudden death, and stroke. Therefore, when retired military personnel and their families reach middle age, there will be an epidemic of obesity-related vascular disease. This will result in the loss of senior personnel and the expertise they contribute to the military. Moreover, the economic burden of cardiovascular disease in active and retired personnel and their families on the military will be enormous. The impact of this trend is being felt now in the military but will accelerate to a very high level over the next 10-20 years if the current trends are left unchecked. In this context, the overall objective of the proposal is to understand at a cellular and molecular level how obesity/insulin resistance promotes atherothrombotic vascular disease. Accomplishing this goal will suggest new targets for drug therapy, which would greatly benefit both the military and the general population. The emphasis of the work is on a key event in advanced atherosclerosis that leads to acute vascular events, namely, advanced lesional macrophage death. Macrophages are the major cell type in atherosclerotic lesions, and when they die and the cell corpses are not rapidly cleared by neighboring phagocytes, necrosis ensues. Plaque necrosis, in turn, promotes plaque disruption and exposure of thrombogenic material. The newly exposed thrombogenic material triggers platelet aggregation (thrombosis), which can acutely obstruct the arterial lumen and cause tissue death (infarction). In the heart, this series of events leads to myocardial infarction and sudden death, and in the brain the consequence is stroke. **Thus, the overall focus of this proposal is advanced lesional macrophage death, and we have excellent progress in the second year of funding in understanding how obesity/insulin resistance can promote this event.**

BODY:

I. Studies related to angiotensin-II (AngII) (Tasks 1-3)

We began with a global hypothesis and preliminary data related to AngII as an important inducer macrophage apoptosis and subsequent plaque necrosis in advanced atherosclerosis. All of our work is based upon a fundamental model of macrophage apoptosis—called the "ER stress-PRR" model (below)--that, based on continuing work in our laboratory, we think is highly relevant to advance atherosclerosis. As we progressed on these studies, it became apparent to us that a fundamental understanding of detailed pathways of macrophage apoptosis in the ER stress-PRR model was required before intelligently and meaningfully approaching the AngII hypotheses. In particular, the known effects of AngII on cellular calcium dynamics and signaling and on a key oxidative enzyme called NADPH oxidase (below) had to be reconciled with our model of macrophage death in advanced atherosclerosis.

A. Tabas lab studies on macrophage apoptosis that have direct impact on AngII signaling—Over the last two years, we have elucidated a macrophage apoptosis model that involves two key elements: endoplasmic reticulum (ER) stress and pattern recognition receptor (PRR) signaling (1-4) (Ref. (4) [Lim *et al*.] is in the Appendix). In exciting work over the last year, we discovered that the ER stress component of this model involves the very pathways that are acted upon by AngII (**Fig. 1**). (For the purposes of this discussion, we will

not delineate in detail the role of PRR signaling, which tips the balance towards apoptosis in ER-stressed macrophages by amplifying STAT1 activation (below) and by suppressing a compensatory cell-survival pathway.)

ER stress triggers the release of ER calcium stores. This event leads to two linked processes that eventually promote apoptosis. In the primary process, calcium release from the ER activates calcium/calmodulin-dependent protein kinases-II (CaMKII), which in turn induces or activates at least five pro-apoptotic processes, all of which are necessary for apoptosis: activation of STAT1; JNK activation; induction of Fas death receptor; generation of reactive oxygen species (ROS); and release of cytochrome c from the mitochondria. In the second process, depletion of calcium in the lumen of the ER, perhaps through dysfunction of calcium-dependent protein chaperones and subsequent protein misfolding, activates the UPR. The UPR effector CHOP induces the enzyme ER oxidase-1α (ERO1α). ERO1a amplifies calcium release from the ER, perhaps by affecting the redox state of IP3 receptors and SERCA. Thus, the role of the UPR *per se* is to amplify the primary signal of increased cytoplasmic calcium. All of these events are necessary for apoptosis as determined by inhibitor, RNAi, or gene-targeting studies (Refs. (3;4) and unpublished data.) Remarkably, most of these pathways are directly impacted by AngII as described in the next section. This new insight has profound implications for our ongoing AngII studies, as described in Section C below).

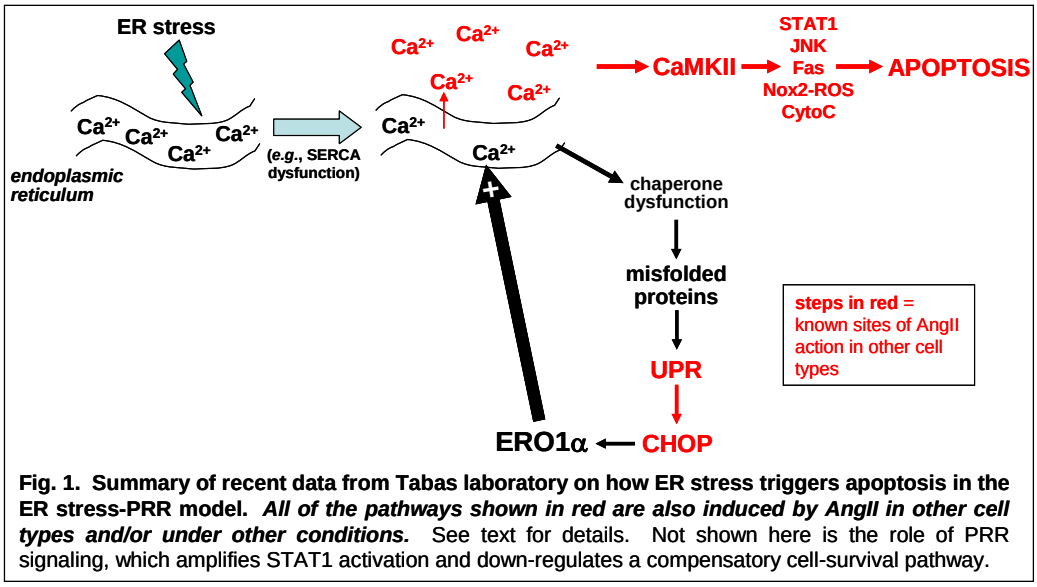

Fig. 1. Summary of recent data from Tabas laboratory on how ER stress triggers apoptosis in the ER stress-PRR model. ***All of the pathways shown in red are also induced by AngII in other cell types and/or under other conditions.*** See text for details. Not shown here is the role of PRR signaling, which amplifies STAT1 activation and down-regulates a compensatory cell-survival pathway.

B. Studies in the literature on AngII signaling that are directly related to new findings in the Tabas laboratory—Important new insight occurred when we compared our new findings on apoptosis signaling in the ER stress-PRR model of advanced lesional apoptosis with signaling pathways triggered by AngII:

1. *ER stress*—Okada *et al*. (5) studied apoptosis of cardiomyocytes in mice subjected to aortic constriction. They found that apoptosis was dependent on a pathway involving UPR activation and induction of CHOP, exactly as in our macrophage model. Remarkably, this UPR-CHOP pathway of apoptosis was induced by and dependent on AngII-AT1R-induced UPR activation *in vivo*.

2. *Intracellular calcium*—In 1982, Reinhart *et al*. (6) reported that AngII could release calcium stores from the ER, thus causing a rise in cytosolic calcium. Since then, there have been similar reports in a variety of cell types, but macrophages have not yet been examined.

3. *CaMKII*—Fern *et al*. (7) were the first to show that AngII activates CaMKII using an adrenal glomerulosa cell model. Most importantly, Palomeque *et al*. (8) recently reported that AngII causes apoptosis in cardiac myocytes through a signaling pathway that is dependent on CaMKII signaling. The authors confirmed that AngII treatment of the cells resulted in activation of CaMKII.

4. *NADPH oxidase*—As mentioned in the original grant application, Griendling *et al*. (9) reported that AngII stimulates NADPH oxidase in vascular smooth muscle cells. AngII-induced superoxide formation was suppressed by an NADPH oxidase inhibitor. Lodha *et al*. (10) showed that an AngII-NADPH oxidase-ROS (reactive oxygen species) pathway was involved in AngII-induced apoptosis in mouse mesangial cells. AngII also activates NADPH oxidase in macrophages (11), and the type of NADPH oxidase in macrophages (Nox2) is particularly sensitive to activation by AngII (12), but no link to apoptosis was made in this cell type.

5. *STAT1*—Marrero *et al*. (13) showed that AngII, acting through AT1R, stimulated STAT1 activation in vascular smooth muscle cells. A similar pathway has been described in cardiomyocytes, where AngII-induced STAT1 activation was shown to be important in AngII-mediated apoptosis in these cells (14). As above, there are no studies in macrophages in this area.

6. *Fas*—Li *et al*. (15) studied ischemia-reperfusion-induced apoptosis in endothelial cells and showed that cell death was enhanced by AngII-AT1R signaling. Remarkably, Fas up-regulation was part of the mechanism of AngII-induced apoptosis in this model.

7. *JNK*—JNK is an important inducer of apoptosis in neurons, and Shenoy *et al*. (16) reported that AngII activated JNK in these cells, leading to JNK-induced apoptosis. Similar reports have since appeared in other cell types, although not macrophages.

8. *Cytochrome c release from mitochondria*—AngII causes apoptosis in endothelial cells. The pathway is characterized by cytochrome c release from mitochondria, and AngII-apoptosis was blocked by an inhibitor of the cytochrome c release pathway, cyclosporin A (17). We found similar results in the ER stress-PRR model of macrophage apoptosis. The role of AngII in this pathway in macrophage apoptosis has not yet been examined.

9. *AngII and atherosclerosis*—Since the submission of this proposal, two separate groups of investigators have transplanted Ldlr-/- or Apoe-/- mice with bone marrow from mice lacking the macrophage AngII receptor, AT1R (18;19). In both cases, atherosclerosis was suppressed by the absence of macrophage AT1R, but there are no studies focusing on advanced lesion macrophage death and plaque necrosis, and none conducted in the setting of obesity and insulin resistance or PPARγ perturbation, as will be carried out in the current grant.

C. Implications—Two important points need to be emphasized to appreciate the implications of our new data in the context of known AngII-induced signaling pathways. First, carefully conducted studies in our own laboratory have shown the effect of AngII on the ER stress-PRR model of macrophage apoptosis is diminished under conditions of robust ER stress. In retrospect, this is exactly what would be predicted if the two pathways were mechanistically redundant. Second, macrophages *in vivo* are unlikely to be subjected to the "sledgehammer" effect of robust ER stress. Rather, as in my most situations *in vivo*, the stressors are likely to be subtle (subthreshold) and multifactorial. Thus, our new data have

led us to consider a critical new concept, namely, that AngII amplifies ER stress-PRR-induced macrophage death under conditions *in vivo* where the ER stress pathway alone is sub-threshold. In the setting of subtle, sub-threshold ER stress *in vivo*, additional stressors like AngII could be rate-limiting for macrophage apoptosis, thus explaining increased plaque necrosis in the setting of a heightened renin-angiotensin system, such as occurs in obesity.

Ongoing studies are now addressing this important new idea by subjecting macrophages to subthreshold ER stress and determining whether AngII can trigger cell death. Moreover, a key aspect of the current model involves ER stress plus one or more other "hits" that tips the balance to apoptosis. Moreover, we can now determine whether AngII can actually substitute for the ER stress hit. Given that ER stress plus PRR activation triggers apoptosis, we will test whether AngII plus PRR signaling can do the same, i.e., can actually substitute for ER stress. In lesions, the implications would be that angII could substitute for ER stress. Moreover, there are potentially critical implications for the interplay between AngII and TZD treatment (below). These new concepts are key to intelligently approaching the pro-atherosclerotic functions of AngII in obesity.

II. PPARγ studies (related to Tasks 3-5)

The fundamental aspects of this project were reported in the previous progress report. At that time, the study required additional plaque analysis and mechanistic experiments. During 2007, the work was submitted to a high-profile heart disease journal, *Circulation*, and much of the year was spent refining the study with new experiments to satisfy the high standards of this journal. We are pleased to report that the study was recently accepted and published in *Circulation* (20). Rather than repeat details that were described in the previous progress report, and because the final publication appears in the Appendix, I will describe below the implications of this study:

Thiazolidinediones (TZDs) are widely prescribed drugs that improve insulin sensitivity in type II diabetics. **Indeed, they are used and studied among military personnel with type 2 diabetes (21;22).** Because insulin resistance is linked to accelerated atherogenesis, it has been proposed that TZDs will be beneficial in decreasing the incidence of atherothrombotic vascular disease. Despite evidence that TZDs reduce early atherosclerotic lesions in mice, their effects on advanced atherosclerosis, which is more relevant to acute coronary syndromes, are not known. The PROactive study failed to show a statistically significant beneficial effect of pioglitazone in diabetic humans on the primary composite endpoint of all-cause mortality, non-fatal myocardial infarction, stroke, acute coronary syndrome, and peripheral vascular disease, although a beneficial effect in the secondary endpoints of all-cause mortality, non-fatal myocardial infarction, and stroke was observed (23). Recent developments have linked the TZD rosiglitazone with a greater risk for heart attack (24). TZDs are drugs that affect a variety of cell types through activation of the nuclear receptor PPARγ as well as through other "off-target" mechanisms. We found that pioglitazone accelerated advanced plaque development in a non-diabetic murine model and that the mechanism appears to be independent of PPARγ. Our overall contention is that while PPARγ-dependent improvement in insulin resistance has the potential to decrease cardiovascular disease in diabetics, this benefit may be optimally realized only if the potential detrimental effects of TZDs, such as those described here, can be eliminated through drug optimization. Given the use of TZDs in general, and pioglitazone in particular, among military

personnel, the implications of this study for diabetes-associated heart disease in the military are substantial.

The completion of this study, in conjunction with the new insights regarding possible sights of AngII action (above), sets the stage in years 3-4 for an informed investigation into the relationship between currently available PPARγ activators (TZDs) and advanced atherosclerosis in the high-AngII setting of obesity, insulin resistance, and type 2 diabetes (**Tasks 3-4**). Pioglitazone promotes apoptosis in macrophages exposed to ER stress (20). Given the common pro-apoptotic signaling between ER stress and AngII (above), it is likely that pioglitazone will also promote apoptosis in AngII-treated macrophages. Thus, pioglitazone may be particularly harmful with respect to macrophage apoptosis in a high-AngII setting such as obesity-related diabetes. These ideas, which have important implications of the treatment of obesity-associated diabetes and drug optimization, will be tested *in vitro* and *in vivo*.

III. Adiponectin studies (Task 6)

We have made an important discovery that impacts the role of adiponectin as a macrophage survival factor. Indeed, we think our new insight can alter the explanation of a number of observations about adiponectin in the literature. We found that adiponectin avidly binds the sepsis-inducing factor, lipopolysaccharide (LPS). This could very well be related to a key structural property of adiponectin, namely its hydrophobic channel (25). Most importantly, we found that many of the effects of adiponectin on cells can be explained by its ability to deliver LPS to cells, perhaps in a unique manner. Based on this new insight, we found that apo-adiponectin (*i.e.*, adiponectin with LPS stripped away) can trigger survival signaling in FC-loaded macrophages by a mechanism still under exploration but that holo-adiponectin (adiponectin-LPS complex) further promotes survival by suppressing the pro-apoptotic branch of the UPR (**Fig. 2**). This latter effect of adiponectin can be mimicked by LPS. The implications of this discovery apply not only to advanced lesional macrophage apoptosis but also to host response to bacterial sepsis, which is mediated by LPS. Of course, the major interest in adiponectin, and its relevance to this grant, is that its levels fall in obesity and obesity-related diabetes. Nonetheless, not only might obesity be a risk factor for increased lesional macrophage death due to a decrease in adiponectin-mediated cell survival and suppression of the UPR, but it may also be a risk factor for response to bacterial sepsis—a known problem with diabetics and, ironically, one that mat exacerbate atherosclerosis (26;27).

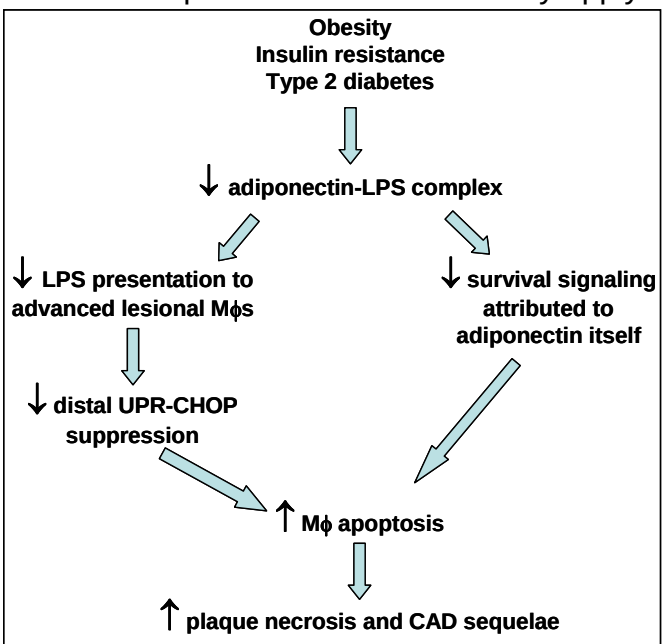

Fig. 2. The dual anti-apoptotic role of adiponectin. The new insight is the role of LPS, which is part of a holo-adiponectin complex, as a UPR-CHOP suppressor. See text for details.

In this context, we have made major progress over the last year at exploring the effects of the adiponectin-LPS branch of our pathway, namely, suppression of the UPR by the LPS moiety. In previous work (see original application), we showed that

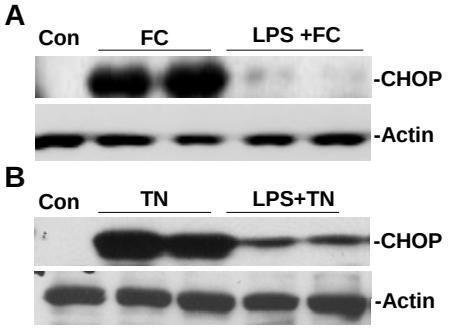

Fig. 3. LPS suppresses UPR-mediated CHOP expression. Mϕs were incubated for 18 h under control (Con) or FC-loaded conditions (***A***) or with the UPR inducer tunicamycin (TN) (***B***) ± 10 ng/ml LPS. Cell extracts were then assayed for CHOP and β-actin as a loading control by immunoblot. The suppression of CHOP is identical to that seen with holo-adiponectin, whereas the suppression was not seen when adiponectin is stripped of LPS.

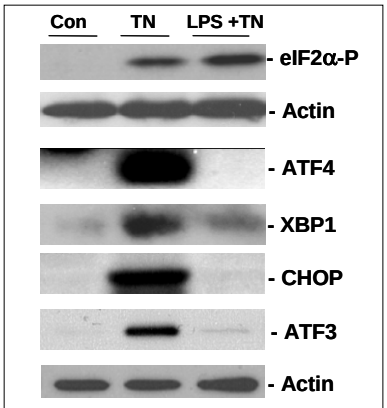

Fig. 4. LPS specifically suppresses the distal UPR. Mϕs were incubated for 10 h under control conditions (Con) or with tunicamycin (TN) ± 10 ng/ml LPS. Cell extracts were then immunoblotted for the above-indicated UPR molecules and β-actin as a loading control. Note that all four distal UPR markers (ATF4, XBP1, CHOP, and ATF3) are suppressed, while the proximal UPR molecule is not. This is exactly the pattern seen with adiponectin.

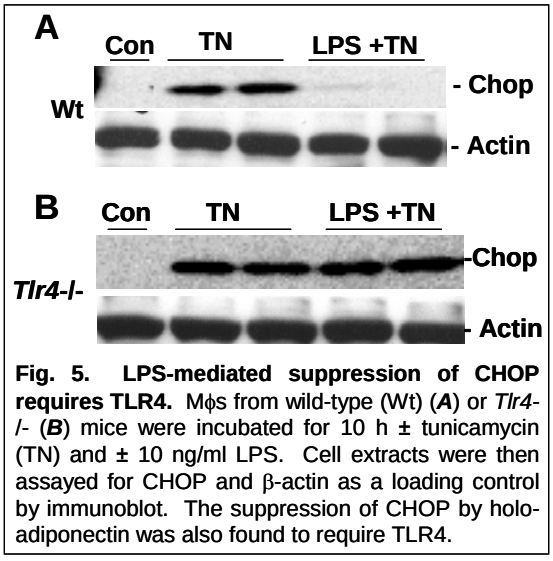

Fig. 5. LPS-mediated suppression of CHOP requires TLR4. Mϕs from wild-type (Wt) (***A***) or *Tlr4-/-* (***B***) mice were incubated for 10 h ± tunicamycin (TN) and ± 10 ng/ml LPS. Cell extracts were then assayed for CHOP and β-actin as a loading control by immunoblot. The suppression of CHOP by holo-adiponectin was also found to require TLR4.

adiponectin is a potent suppressor of FC-induced CHOP. We then found that this was a unique property of holo-adiponectin and that apo-adiponectin (stripped of LPS) did not suppress the distal UPR. To explore this further, we showed that LPS itself is a potent suppressor of FC- and tunicamycin-induced CHOP expression (**Fig. 3**). A unique property of adiponectin-mediated suppression of the UPR is that it suppresses the distal UPR (ATF4, XBP1, CHOP, and ATF3) but not the proximal UPR (IRE1, PERK, eIF2α-P). Consistent with this being mediated by the LPS moiety of holo-adiponectin, this is exactly what we found with LPS (**Fig. 4**).

Also as found with adiponectin, the ability of LPS to suppress the distal UPR was dependent on TLR4 (**Fig. 5**). TLR4 signals through two pathways, each controlled by an adaptor pair. One involves the adaptors MyD88 and Mal, and the other uses TRIF and TRAM as adaptors. The MyD88 pathway is the one usually associated with TLR4 signaling, including TLR4-induced NFκB activation. Remarkably, the ability of LPS to suppress the distal UPR was mediated by the TRIF/TRAM pathway, not the MyD88 pathway (**Fig. 6A-C**). This conclusion was supported by showing that the response could be mimicked by poly-IC, a ligand for TLR3, which signals only through TRIF/TRAM, but not by peptidoglycan, a ligand for TLR2, which signals only through MyD88 (**Fig. 6D**).

Most importantly, we were able to show that LPS suppressed the distal UPR *in vivo* by the same mechanism. As shown in **Fig. 7**, when mice were pre-treated with low-dose LPS and then subsequently challenged with tunicamycin, there was marked suppression of CHOP in both the kidney and liver. We found that this suppression did not occur when *Trif;Tram-/-* mice were used. Moreover, we have been able to verify these in vivo results using a more physiologic inducer of CHOP, namely, high-dose LPS. Thus, mice pre-treated with low-dose LPS have show decreased induction of CHOP in the kidney and liver when challenged with high-dose LPS.

Having now elucidated, through a reductionist approach, the mechanism of LPS-mediated suppression of the UPR, we are now poised to extend these studies in years #3-4 to holo-adiponectin, *i.e.*, adiponectin-LPS complex. We will explore both holo- and apo-adiponectin in cultured macrophages, as planned, in terms of apoptosis, cytokine production, and effects on AngII and PPARγ on these processes. Regarding *in vivo* studies, we will first determine whether adiponectin

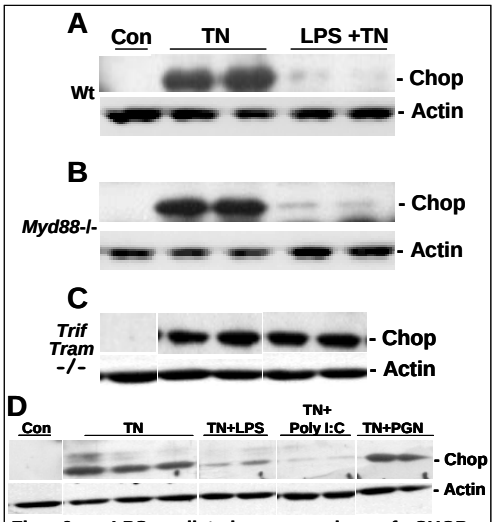

Fig. 6. LPS-mediated suppression of CHOP requires the TRIF/TRAM pathway of TLR4 signaling. Mϕs from wild-type (Wt) (***A, D***), *Myd88-/-* (***B***), or *Trif;Tram-/-* (***C***) mice were incubated for 10 h ± tunicamycin (TN) and ± 10 ng/ml LPS or ± poly-IC or peptidoglycan (PGN). Cell extracts were then assayed for CHOP and β-actin as a loading control by immunoblot.

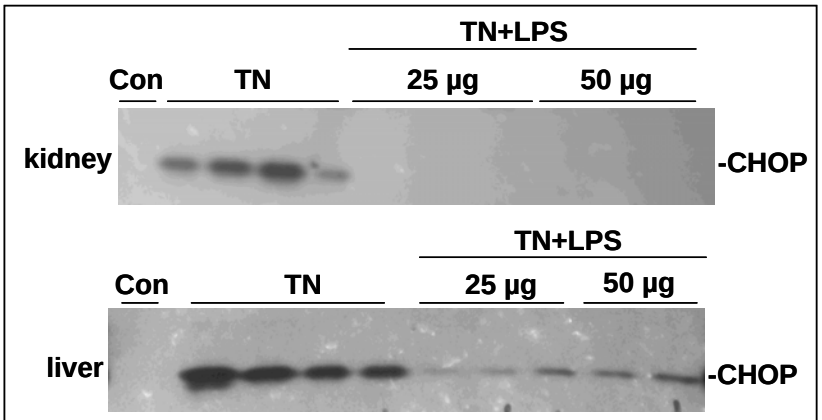

Fig. 7. LPS suppresses CHOP *in vivo*. Mice were pre-treated ± 25 or 50 µg LPS for 18 h. They were then injected with tunicamycin or vehicle control (Con), and then 2 h later the kidney and liver were harvested and immunoblotted for CHOP.

is complexed with adiponectin *in vivo* and then determine *in vivo*, as originally planned, whether adiponectin in an LPS-dependent manner, suppresses macrophage apoptosis and plaque necrosis in *ob/ob*;*Ldlr*-/- mice.

In summary, critical work during year #2 has revealed a novel aspect of adiponectin signaling that is likely to have profound implications not only for the studies herein but also for other aspects of LPS biology in obesity, where adiponectin is decreased. With regard to the focused Tasks of this proposal, this information is essential for proper design and execution of further mechanistic studies with cultured macrophages and pathophysiologic studies *in vivo* on obesity-related atherosclerosis. Thus, in years 3-4 of the proposal, we will integrate this new knowledge with the UPR-independent survival effects of apo-adiponectin in terms of advanced atherosclerosis, as originally planned.

IV. UPR activation in advanced human atherosclerotic lesions (Task 7)

During the past year, Myoishi *et al*. (28) have shown that vulnerable human plaques, but not benign ones, have evidence of robust UPR activation and that this correlates not only with plaque vulnerability but also with intimal cell apoptosis. This is exactly congruent with the hypothesis we first conceived in our 2003 *Nature Cell Biology* paper (1). Over the last year, we took this observation one step further by showing that a key mechanistic step downstream of the UPR is activated in human advanced but not early atherosclerotic lesions. As explained above, the key downstream pro-apoptotic pathway of UPR activation is release of ER calcium into the cytosol, with subsequent activation of CaMKII. One of the most important pro-apoptotic molecules downstream of CaMKII is STAT1 (4) (see Appendix; Lim *et al*., *Circulation* in press). In particular, STAT1 is serine-phosphorylated, which in turn directly lead to induction of apoptosis. In collaboration with Dr. Renu Virmani (see original application, Task 8) and her colleague Dr. Frank Kolodgie, we showed for the first time that Ser-P-STAT1 was present in thin-capped atheromata from human coronary arteries but not in more benign lesions (**Fig. 8**). Ser-P-STAT1 expression occurs in macrophage-rich and apoptotic (TUNEL)-rich areas near necrotic cores (**Fig. 8C**). These data are the first to show that a critical pro-apoptotic mediator of UPR-induced macrophage apoptosis is expressed in advanced human plaques.

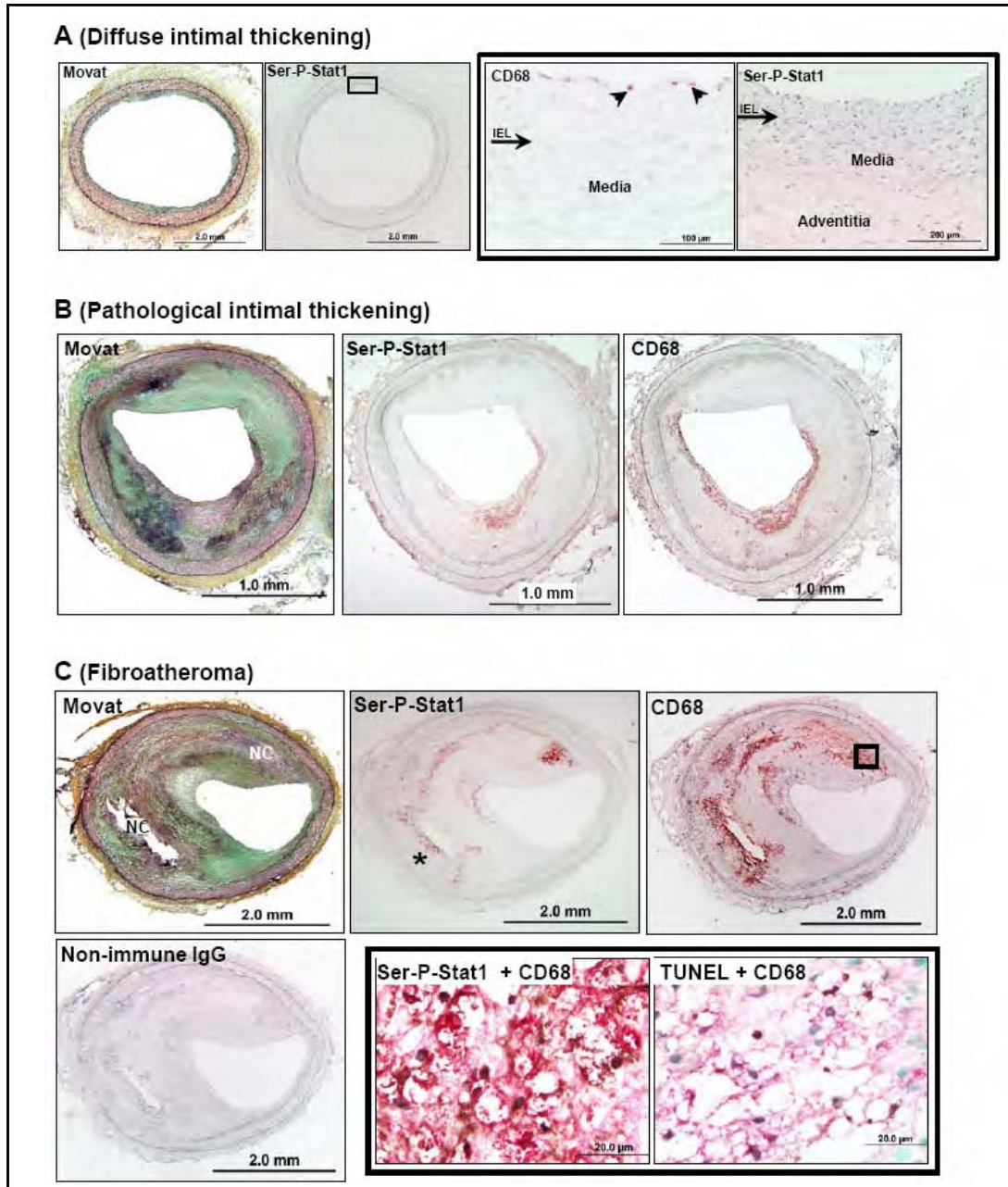

Figure 8. Ser-P- STAT1 is present in advanced human coronary atheromata but not in diffuse intimal thickening. The sections were stained with Movat pentachrome, anti-Ser-P-STAT1, anti-CD68, and non-immune IgG, as indicated in the images. (***A***) Diffuse intimal thickening. The CD68 and Ser-P-STAT1 images on the right are higher magnifications of the area indicated by the box in the low-magnification Ser-P-STAT1 image. As shown in the higher magnification images, only a few CD68-positive macrophages are present directly under the endothelium (*arrowheads*). Ser-P-STAT1 was not detected. *Arrow* indicates internal elastic lamina (*IEL*). (***B***) Pathological intimal thickening. Ser-P-STAT1 staining coincides with CD68-positive macrophages. (***C***) Fibroatheroma: Ser-P-STAT1 staining coincides with CD68-positive macrophages. Some of the Ser-P-STAT1 staining is in macrophages surrounding a necrotic area (*asterisk*). The lower middle and right images are higher magnifications of the area indicated by the box in the low-magnification CD68 image. These lower middle image shows the result of double immunostaining with anti-Ser-P-STAT1 (*dark, punctate structures*) and anti-CD68 (*red*), demonstrating Ser-P-STAT1 in the nuclei of macrophages. The lower right image shows the result of double immunostaining with TUNEL (*dark, punctate structures*) and anti-CD68 (*red*), demonstrating apoptotic macrophages. Nuclei of non-apoptotic cells are stained green. Note that exact alignment of the nuclei is not possible due to the fact that the sections are from separate tissue slices.

KEY RESEARCH ACCOMPLISHMENTS IN YEARS 1-2 AND PLANS FOR YEARS 3-4:

Task #	Year 1	Year 2	Planned Years 3-4
1	• Initiation of key signaling studies that we reasoned would be critical for understanding the role of AngII in Mϕ apoptosis.	• Elucidation of the key signaling pathways involved in the ER stress-PRR model of apoptosis, with an emphasis on those pathways known to be direct targets of AngII, namely, calcium-CaMKII-NADPH oxidase-ROS. This work resulted in a high-profile manuscript recently accepted by *Circulation*.	• We will now focus on the specific pro-apoptotic signaling pathways elucidated in Year 2, with the hypothesis that AngII promotes death under sub-threshold conditions of ER stress and/or as a substitute for ER stress in the ER stress-PRR model of Mϕ apoptosis.
2			• The strategies described above for Task 1 will be used to explore the role of AngII in cytokine production.
3	• Elucidation of the role of TZDs on advanced lesional Mϕ death.		• Because we now know that TZDs have off-target effects, we need to directly manipulate PPARγ *in vivo*, which we will do using the Cre-lox model described in our Circulation accepted paper. Therefore, we will address this Task by determining whether the proposed detrimental effects of AngII on advanced atherosclerosis are amplified in Mf-specific PPARγ KO mice.
4		• The TZD study was refined with more in-depth mechanism and completion of in vivo studies, resulting in submission and publication by *Circulation*.	• We will use *ob/ob*:*Ldlr*-/- mice to test the effects of both AngII and PPARγ on advanced atherosclerosis, based on the principles outlined in Tasks 1-3.
5			
6	• Mechanistic studies on how adiponectin suppresses the UPR.	• Elucidation both *in vitro* and *in vivo* that the key UPR-suppressive action of adiponectin is carried out by an adiponectin-LPS complex (holo-adiponectin) through a unique TLR-TRIF/TRAM pathway.	• We will explore both holo- and apo-adiponectin in cultured macrophages, as planned, in terms of apoptosis, cytokine production, and effects on AngII and PPARγ on these processes. Regarding *in vivo* studies, we will first determine whether adiponectin is complexed with adiponectin *in vivo* and then determine *in vivo*, as originally planned, whether adiponectin in an LPS-dependent manner, suppresses macrophage apoptosis and plaque necrosis in *ob/ob*;*Ldlr*-/- mice.
7	• Identification of key downstream UPR effectors as candidates to explore in human lesions.	• First demonstration that a key pro-apoptotic signaling molecule downstream of the UPR—Ser-P-STAT1—is expressed in advanced but not early human coronary artery lesions. (Myoishi et al. reported that advanced human lesions express UPR markers, which was correlated with apoptosis and plaque vulnerability.)	• We will continue our collaboration with Dr. Virmani and further probe human lesions for the key signaling molecules of UPR-induced apoptosis, including CaMKII and NADPH oxidase.

REPORTABLE OUTCOMES:

- Two publications in the high-profile journal *Circulation* (impact factor > 10)
    - Thorp E, Kuriakose G, Shah YM, Gonzalez FJ, Tabas I. Pioglitazone increases macrophage apoptosis and plaque necrosis in advanced atherosclerotic lesions of nondiabetic low-density lipoprotein receptor-null mice. *Circulation*. 2007 116(19):2182-90.
    - Lim WS, Timmins JM, Seimon, TA, Sadler, A, Kolodgie FD, Virmani R, Tabas I. STAT1 is Critical for Apoptosis in Macrophages Subjected to Endoplasmic Reticulum Stress in Vitro and in Advanced Atherosclerotic Lesions in Vivo. *Circulation*, In press.

- Book chapter: Tabas, I., Seimon, T. Arrelano, J. Li, Y, Forcheron, F., Cui, D., Han, S., Liang, C.P., Tall, A., and Accili, D. (2007) The impact of insulin resistance on macrophage death pathways in advanced atherosclerosis. IN *Fatty Acids and Lipotoxicity in Obesity and Diabetes*. Novartis Foundation Symposium 286. John Wiley & Sons, Ltd., Chichester, UK, pp. 99-112.

- Abstract for annual Drugs Affecting Lipid Metabolism meeting in New York City, October, 2007

- Dr. Dongying Cui is assembling her data on holo-adiponectin-LPS-mediated UPR suppression (presented above) into a manuscript

- Oral presentations on the material described in this progress report

- Post-doctoral training related to the aforementioned projects in the Tabas laboratory

CONCLUSIONS:

During year #2, we gained new and critical information for our understanding the cellular-molecular links between obesity and atherothrombotic vascular disease. With regard to Tasks 1-2, we found that the key signaling pathways involved in the ER stress-PRR pathway of advanced lesional apoptosis are precisely those that are affected by AngII. This new insight provides a feasible mechanistic explanation for some of our previous results with AngII and now sets the stage to address the role of AngII in macrophage apoptosis, both in vitro and in vivo, in a new and more focused context. With regard to PPARg, we completed and published our study on TZDs, thus reporting to the cardiovascular community that a potential adverse effect of TZDs could be related to advanced lesional macrophage apoptosis. This work may explain some recent clinical data on adverse effects of TZDs and sets the stage for the design of better drugs to treat obesity-related diabetes and heart disease. Moreover, these studies set the stage for experiments looking at the interaction between AngII and PPARγ. Our adiponectin studies focused on a newly discovered property of this hormone, namely, its ability to bind and deliver LPS to cells. This property is responsible for the UPR-suppressive effects of holo-adiponectin and provide key new insight necessary to continue our mechanistic and in vivo studies related to the decline of this

hormone in obesity. Finally, the large field of cardiovascular researchers is beginning to appreciate the potential role of the UPR in human plaque vulnerability through probing of human coronary artery lesions. We provided an important component of this effort by showing that a key signaling molecule involved in UPR-induced apoptosis is activated in advanced but not early human lesions. In summary, we have made substantial progress in understanding how obesity leads to accelerated heart disease at a molecular-cellular level by the elucidation of novel pathways of macrophage apoptosis in advanced atherosclerosis. Continuing work on the Tasks of this grant will hopefully suggest hew therapeutic approaches to this evolving epidemic in the military and in the general society.

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APPENDICES:

- **Published *Circulation* paper (Thorp *et al*.)**
- **In-press *Circulation* manuscript (Lim *et al*.)**
- **Book chapter (Fatty Acids and Lipotoxicity in Obesity and Diabetes) (Tabas *et al.*)**
- **DALM abstract (Tabas *et al.*)**
- **PI's Curriculum vitae**

Pioglitazone Increases Macrophage Apoptosis and Plaque Necrosis in Advanced Atherosclerotic Lesions of Nondiabetic Low-Density Lipoprotein Receptor Null Mice

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Pioglitazone Increases Macrophage Apoptosis and Plaque Necrosis in Advanced Atherosclerotic Lesions of Nondiabetic Low-Density Lipoprotein Receptor–Null Mice

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Background—Thiazolidinediones (TZDs), which have actions that involve both peroxisome proliferator–activated receptor (PPAR)-γ–dependent and –independent effects, improve insulin sensitivity in type II diabetes and inhibit early atherogenesis in mice. However, the effects of TZDs on advanced lesion progression are unknown.

Methods and Results—Pioglitazone and rosiglitazone enhanced macrophage apoptosis by a number of stimuli, including those thought to be important in advanced atherosclerosis. Macrophage death was not enhanced by non-TZD PPARγ activators, and TZD-induced apoptosis was still observed in PPARγ-deficient macrophages. In wild-type macrophages, death enhancement was associated with reduced activation of the cell-survival mediator nuclear factor-κB. TZDs also increased the ability of macrophages to phagocytically clear apoptotic cells, which is proposed to protect against plaque necrosis in advanced lesions. The mechanism of this effect was complex, involving both PPARγ-dependent and -independent mechanisms. To explore the net effect on advanced atherosclerosis in vivo, $Ldlr^{-/-}$ mice were fed a nondiabetogenic cholesterol-enriched diet to promote midstage lesions. Then, pioglitazone was administered with the diet for an additional 10 weeks. Aortic root lesions from the pioglitazone-treated mice showed a substantial increase in apoptotic cells and plaque necrosis compared with lesions from non–drug-treated mice.

Conclusions—The potential atheroprotective effects of TZDs conferred by insulin sensitization may be partially offset by adverse effects on advanced atherosclerosis. Because the mechanisms of the beneficial and proposed adverse effects may differ, these findings have potentially important implications for drug optimization. **(*Circulation*. 2007;116:2182-2190.)**

Key Words: apoptosis ■ atherosclerosis ■ macrophages ■ plaque ■ drugs

Thiazolidinediones (TZDs) are drugs that affect cells through activation of the nuclear receptor peroxisome proliferator-activated receptor (PPAR)-γ and through other "off-target" mechanisms.[1] In patients with type II diabetes mellitus, TZDs improve insulin sensitivity, an effect ascribed to PPARγ activation.[2] Because insulin resistance promotes atherogenesis,[3] it has been proposed that TZDs will be beneficial in decreasing the incidence of atherothrombotic vascular disease. In this context, a number of animal studies have shown antiatherogenic effects of TZDs, although the mechanisms are not always correlated with improved metabolic parameters.[4–8] Interestingly, other animal studies have failed to show an antiatherogenic effect of TZDs even in the setting of improved insulin sensitivity.[9] In humans, pioglitazone failed to show a statistically significant beneficial effect on a primary composite end point of all-cause mortality and cardiovascular disease in the Prospective Pioglitazone Clinical Trial in Macrovascular Events (PROactive).[10] Moreover, in a recent meta-analysis of rosiglitazone trials in diabetic patients, the drug was associated with a 43% increase in myocardial infarction and a 64% increase in cardiovascular death.[11] On the other hand, pioglitazone was found to have an overall beneficial effect in diabetic patients on the individual endpoints of all-cause mortality, nonfatal myocardial infarction, and stroke in both the PROactive study and in a recent meta-analysis by Nissen and colleagues.[10,12]

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The above findings highlight the uncertainty related to the overall mechanisms and consequences of TZDs on atherothrombotic disease. Although one might argue, on the basis of the aforementioned meta-analyses, that rosiglitazone has adverse effects on coronary disease that are unique to that 1 compound,[11,12] the effects of pioglitazone on advanced atherosclerosis are far from settled. Regarding potential antiatherogenic

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The online-only Data Supplement can be found with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.107.698852/DC1.

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mechanisms, insulin sensitization involves PPARγ activation in the liver, adipose, and muscle.[2,3] On the other hand, PPARγ is expressed in atherosclerotic lesional cells, including macrophages.[3] Whereas TZDs were found to increase expression of the oxidized low-density lipoprotein (LDL) receptor CD36 in cultured macrophages,[13] TZDs reduced CD36 expression and uptake of oxidized LDL in a mouse model of insulin resistance, concomitant with improved insulin signaling in macrophages.[14] Furthermore, activation of PPARγ has been shown to enhance macrophage cholesterol efflux through transcriptional induction of LXRα and perhaps ABCA1[5,15] and to suppress proinflammatory cytokine secretion from activated macrophages.[16]

To fully understand how TZDs might affect atherothrombotic disease, it is important to consider how these drugs might affect specific processes that promote advanced plaque progression. Two such processes are advanced lesional macrophage death and the phagocytic clearance of these apoptotic cells (efferocytosis). Macrophage apoptosis is increased in advanced lesions and can lead directly to plaque necrosis when these apoptotic cells are not efficiently cleared by neighboring macrophage phagocytes.[17] Plaque necrosis, in turn, promotes plaque disruption and subsequent acute thrombosis.[18] In vivo studies suggest that 1 mechanism of macrophage death unique to advanced lesions is that triggered by an excess of intracellular unesterified, or "free," cholesterol (FC) delivered by atherogenic lipoproteins.[19] The lipoproteins and FC trigger a series of proapoptotic signal transduction pathways involving the type A scavenger receptor, toll-like receptor 4, the mitogen-activated protein kinase JNK, and the endoplasmic reticulum (ER) stress pathway known as the unfolded protein response (UPR).[20,21] Although the mechanisms of defective efferocytosis in advanced lesions are not known, possibilities include competitive inhibition of apoptotic cell-phagocyte interaction by oxidized lipoproteins and suppression of apoptotic cell engulfment by oxidative stress and hypoxia.[17]

In this context, we report here that TZDs enhance macrophage apoptosis induced by a number of stimuli and promote efferocytosis of apoptotic cells. Most important, when pioglitazone is administered to nondiabetic *Ldlr*$^{-/-}$ mice after midstage lesions have already been established, the net effect is increased advanced lesional macrophage apoptosis and plaque necrosis. Because the mechanisms of the beneficial and proposed adverse effects of TZDs may differ, these findings have potentially important implications for drug optimization.

Methods

See the online-only Data Supplement for an expanded Methods section.

Mice

Wild-type macrophages were obtained from 8- to 10-week-old female C57Bl6/J mice (The Jackson Laboratory, Bar Harbor, Me). For the PPARγ-deficient studies, macrophages were from 8- to 10-week-old female PPARγ$^{fl/fl}$×LysMCre mice (PPARγ$^{\Delta M\varphi}$), which have deficient PPARγ expression, or from control PPARγ$^{fl/fl}$ mice, which have normal PPARγ expression.[22] The PPARγ$^{\Delta M\varphi}$ and PPARγ$^{fl/fl}$ mice are on the C57Bl/6N-FVB genetic background. *Ldlr*$^{-/-}$ mice on a C57Bl/6J background were purchased from Jackson Laboratories.

Macrophage Incubations and Apoptosis Assays

Before FC loading, macrophages were preincubated with TZDs in dimethyl sulfoxide or dimethyl sulfoxide vehicle control for 18 to 24 hours as indicated. The macrophages were FC loaded by incubation with 100 μg/mL acyl-LDL plus 10 μg/mL 58035 (to inhibit acetyl-coenzyme A acetyltransferase–mediated cholesterol esterification). Externalization of phosphatidylserine, a sign of early to midstage apoptosis, was detected by quantitative microscopy and flow cytometry with Alexa-488–labeled annexin V (Molecular Probes, Carlsbad, Calif). Membrane leakiness, a sign of late-stage apoptosis, was detected by staining with propidium iodide. Micrographs were captured with an Olympus IX-70 inverted fluorescence microscope, and 5 representative fields (≈1000 cells total) per condition were used to quantify the number of annexin V–positive, propidium iodide–positive, and total cells. For flow cytometry, macrophages were rinsed in cold PBS, resuspended in annexin V–binding buffer (10 mmol/L HEPES, pH 7.4, 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$), and stained with Alexa-488–labeled annexin V for 15 minutes. Cells were then rinsed with binding buffer and subjected to flow cytometry as previously described.[23]

Efferocytosis Assay

Efferocytosis was assessed as previously described[24] with minor modifications. The source of apoptotic cells, which were prelabeled with the green fluorescent dye calcein AM, was FC-loaded peritoneal macrophages or ultraviolet-treated J774 murine macrophages (15 minutes at 254 nm, 20 J/cm^2). Before inducing apoptosis, the macrophages were fluorescently labeled with calcein AM (green) (Molecular Probes). The apoptotic cells were overlaid onto monolayers of octadecylrhodamine-labeled (red) macrophages (phagocytes) at a 1:1 ratio. After 30 to 45 minutes, noningested apoptotic macrophages were removed by vigorous agitation and rinsing. The adherent cells were then fixed in paraformaldehyde and viewed and imaged by fluorescence microscopy. These images were used to quantify phagocytic uptake, which was distinguished from external apoptotic cell-phagocyte binding by confocal microscopy.

Pioglitazone-Atherosclerosis Study

At 6 weeks of age, *Ldlr*$^{-/-}$ mice were fed a gamma-irradiated, low-fat (10-kcal fat), high-cholesterol (0.5% or 5.3 g cholesterol/4057 kcal) semisynthetic (AIN76) Clinton/Cybulsky pellet diet (D00083101) from Research Diets (New Brunswick, NJ).[25] The mice were maintained on this diet for 8 weeks at ≈3 g/d. The mice were then split into 2 groups; 1 group received pioglitazone in addition to the semisynthetic diet for an additional 10 weeks. According to food intake, the dose of pioglitazone was 40 mg/kg body weight per day. All animal protocols were approved by the Columbia University Institutional Animal Care and Use Committee.

Lesion Analysis and Immunohistochemistry

For morphometric lesion analysis, sections were stained with Harris' hematoxylin and eosin. Total intimal lesion area (between internal elastic lamina to the lumen) and acellular/anuclear areas (negative for hematoxylin-positive nuclei) per cross section were quantified by taking the average of 6 sections spaced 30 μm apart beginning at the base of the aortic root. Histomorphological analysis of collagen was performed with Masson's trichrome stain (Richard-Allan Scientific, Kalamazoo, Mich) and elastin stain (hematoxylin-iodine-ferric chloride, Sigma Chemical Co, St Louis, Mo). Images were viewed and captured with a Nikon Labophot 2 microscope equipped with a Sony CCD-Iris/RGB color video camera attached to a computerized imaging system with Image-Pro-Plus 3.0 software. For immunohistochemistry, antigens were retrieved via heating in an EDTA solution, followed by hydrogen peroxide/methanol blocking of endogenous peroxidase. Blocking was performed with immunoglobulin from the species of the secondary antibody. Macrophages were detected with a rabbit anti-macrophage antibody (AIA31240) from Accurate Chemical and Scientific Corporation (Westbury, NY). Smooth muscle cell actin was detected with Zymed's mouse anti-smooth muscle actin (1A4) following the protocol of Zymed's Histomouse-SP Kit (Invitrogen, Carlsbad, Calif). Secondary antibodies were biotinylated conjugates that were subsequently detected with streptavidin–horseradish peroxidase. The horseradish peroxidase substrate was diaminobenzidine. Images were viewed and captured as above. Apoptotic cells in atherosclerotic lesions were detected by

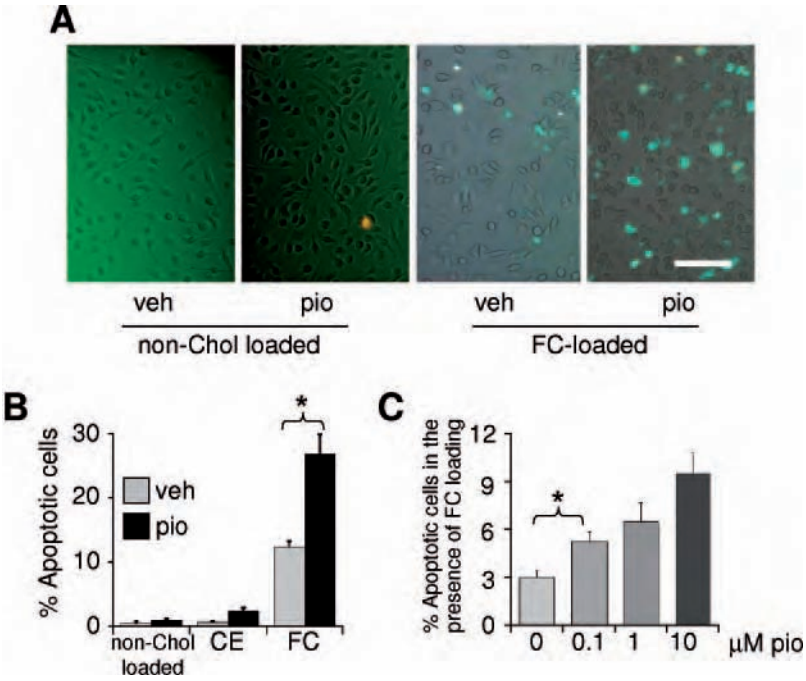

Figure 1. Pioglitazone enhances FC-induced macrophage apoptosis. A, Macrophages were pretreated for 18 hours with 10 μmol/L pioglitazone (pio) or vehicle (veh) control and then incubated under nonloading or FC-loading conditions for an additional 12 hours with or without pioglitazone or vehicle control. The cells were then stained with annexin V (green) and propidium iodide (red) and viewed by fluorescence microscopy. Bar=100 μm. B, Quantification of annexin V–positive, propidium iodide–negative (apoptotic) macrophages in nonloaded, cholesteryl ester (CE)–loaded, or FC-loaded macrophages with or without pioglitazone. C, Percent of apoptotic FC-loaded macrophages at the indicated doses of pioglitazone. Chol indicates cholesterol. *$P<0.05$.

Tdt-mediated dUTP nick-end labeling (TUNEL) after proteinase K treatment using the TMR-red kit from Roche (Nutley, NJ). The stringency methods of Kockx[26] were followed to avoid nonspecific staining. Nuclei were counterstained with Hoechst for 5 minutes. The slides were viewed and imaged by fluorescent microscopy. For quantitative data analysis, the stained areas in the images were obtained and quantified as described above.

Statistical Analysis

Data are presented as mean±SEM. The absence of error bars in the bar graphs signifies that SEM values were smaller that the graphic symbols. For paired groups, Student's *t* test was used. ANOVA was used for >2 groups, and multifactor ANOVA was used under conditions of ≥2 independent variables. The post hoc analysis was the Tukey procedure.

Results

TZDs Enhance Macrophage Apoptosis Induced by FC Loading and Other Inducers

To determine the effects of TZDs on macrophage apoptosis in a context relevant to advanced atherosclerosis, macrophages were preincubated for 18 hours in the absence or presence of 10 μmol/L pioglitazone and then incubated for an additional 12 hours under control or FC-loading conditions with or without pioglitazone. The cells were then assayed for apoptosis with annexin V staining. As shown in Figure 1A and 1B, pioglitazone treatment led to a ≈2-fold increase in FC-induced apoptosis. A similar increase in apoptosis was measured by annexin V flow cytometry (see Figure I of the online-only Data Supplement). Pioglitazone did not induce de novo apoptosis in macrophages that were not cholesterol loaded or in cholesteryl ester–loaded macrophages, which represent the state of most macrophages ("foam cells") in early atherosclerotic lesions (Figure 1B). Death enhancement by pioglitazone followed a direct dose-response relationship, with apoptosis enhancement observed even at the lowest dose of 100 nmol/L (Figure 1C). One possible mechanism for the enhancement of FC-induced apoptosis by TZDs could be increased lipoprotein uptake, leading to increased delivery of lipoprotein-derived FC to the ER. The latter processes induced the UPR effector CCAAT/enhancer-binding protein-homologous protein (CHOP), which is required for apoptosis.[27] However, we found that pioglitazone pretreatment did not increase the uptake and processing of [^{125}I]acetyl-LDL, the delivery of acetyl-LDL cholesterol to the ER, or the expression of CHOP (data not shown). Moreover, we found that pioglitazone was able to enhance macrophage apoptosis induced by 2 noncholesterol factors, the UPR activator thapsigargin and the protein phosphatase inhibitor staurosporine (Figure 2A and 2B). Thus, pioglitazone is a general enhancer of macrophage apoptosis and does not depend on FC loading per se.

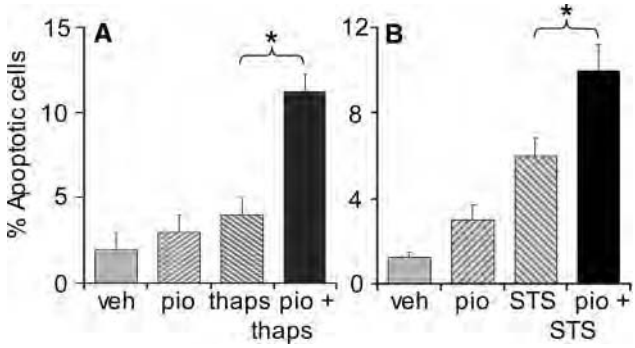

Figure 2. Pioglitazone increases apoptosis in both ER and non-ER stressed macrophages. A, Macrophages were pretreated for 24 hours with 10 μmol/L pioglitazone (pio) or vehicle (veh) control and then incubated for an additional 15 hours with 10 μmol/L pioglitazone, 2.5 mmol/L thapsigargin (thaps), or both reagents. The cells were then stained with annexin V (green) and propidium iodide (red), viewed by fluorescence microscopy, and quantified for apoptosis. B, The same procedure was followed except that the macrophages were treated for 11 hours with 100 nmol/L staurosporine (STS) instead of thapsigargin where indicated. *$P<0.05$.

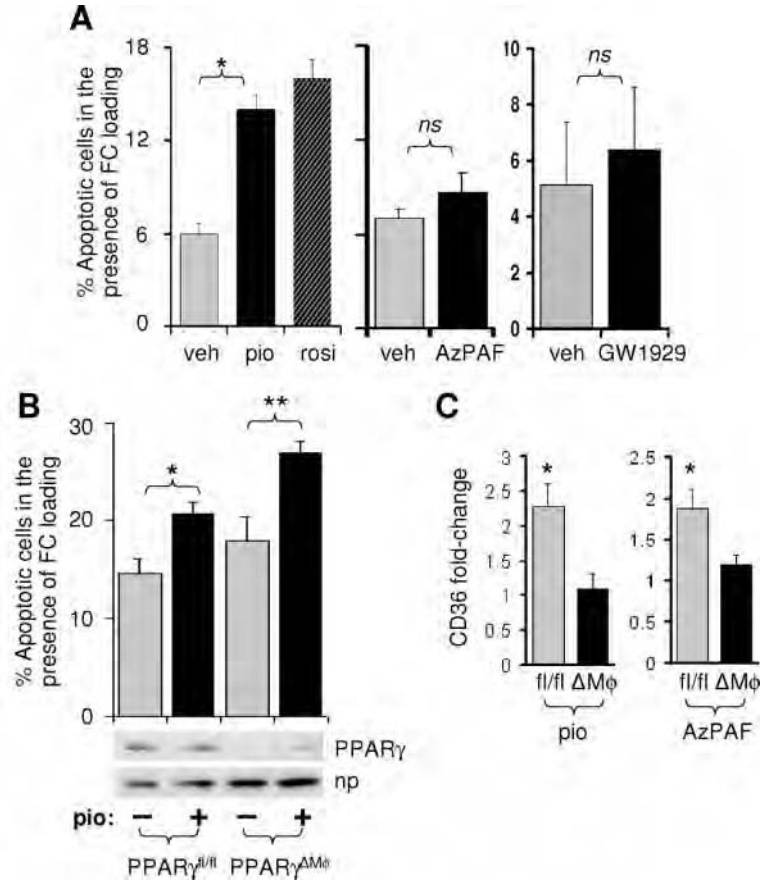

Figure 3. Enhancement of FC-induced macrophage apoptosis is not seen with a non-TZD PPARγ activator, and TZD-enhanced apoptosis occurs normally in PPARγ-depleted macrophages. A, Percent of apoptotic FC-loaded macrophages after treatment with 10 μmol/L pioglitazone (pio), 5 μmol/L rosiglitazone (rosi), 5 μmol/L AzPAF, 1 μmol/L GW1929, or vehicle control (veh). B, Peritoneal macrophages from PPARγ$^{fl/fl}$ and PPARγ$^{\Delta M\varphi}$ mice were FC loaded after pretreatment with 10 μmol/L pioglitazone or vehicle control and then assayed and quantified for apoptosis. The immunoblot below the graph shows nuclear PPARγ (≈57 kDa) and nucleophosmin (np), a nuclear protein that serves as a loading control. C, Fold increase in CD36 mRNA by pioglitazone and AzPAF versus vehicle control in macrophages from PPARγ$^{fl/fl}$ and PPARγ$^{\Delta M\varphi}$ mice. Data are derived from quantitative polymerase chain reaction measurements of CD36 mRNA relative to 36B4 mRNA. *$P<0.05$ vs non–drug-treated control.

Macrophage Death Is Not Enhanced by Non-TZD PPARγ Activators, and TZD-Enhanced Apoptosis Is Observed in PPARγ-Deficient Macrophages

TZDs can affect cells through both PPARγ-dependent and -independent mechanisms.[1] To determine whether death enhancement was specific to pioglitazone or TZDs in general, we measured FC-induced apoptosis in the presence of the TZD rosiglitazone and the non-TZD PPARγ ligands and agonists azelaoyl PAF (AzPAF) and GW1929.[28,29] Enhancement of FC-induced apoptosis was observed with rosiglitazone but not with either AzPAF or GW1929 (Figure 3A). To verify that AzPAF in particular was acting as a PPARγ agonist in our system, we showed that mRNA for the PPARγ-dependent gene CD36 was induced 1.8-fold in the AzPAF-treated macrophages (Figure 3C). AzPAF also increased levels of adipose differentiation–related protein, an PPARγ-inducible protein (data not shown). These data raised the possibility that apoptosis enhancement was being mediated through a PPARγ-independent effect of TZDs. To further explore this possibility, we used peritoneal macrophages from mice carrying floxed alleles of PPARγ with (PPARγ$^{\Delta M\varphi}$) or without (PPARγ$^{fl/fl}$) the macrophage-targeted LysMCre recombinase gene.[22] As expected, macrophages from PPARγ$^{fl/fl}$ mice expressed PPARγ, whereas those from PPARγ$^{\Delta M\varphi}$ mice had undetectable or very low PPARγ expression (Figure 3B, immunoblot). Moreover, expression of CD36 mRNA was unchanged after pioglitazone or AzPAF treatment of PPARγ$^{\Delta M\varphi}$ macrophages compared with an ≈2-fold induction in control PPARγ$^{fl/fl}$ cells (Figure 3C). As shown by the quantified apoptosis data in Figure 3B, pioglitazone enhanced FC-induced apoptosis in both PPARγ-expressing and PPARγ-deficient macrophages. These data, together with the AzPAF and GW1929 data above, suggest that enhancement of macrophage apoptosis by TZDs occurs via a PPARγ-independent mechanism.

Pioglitazone Suppresses Nuclear Factor-κB-p65, a Cell-Survival Factor, in FC-Loaded Macrophages

We next sought to probe the mechanism of pioglitazone-induced enhancement of apoptosis. We previously reported that FC loading activates the nuclear factor (NF)-κB pathway in macrophages through a mechanism that involves both ER stress and toll-like receptor 4.[21,30] NF-κB activation can drive prosurvival responses in many cell types,[31] and TZDs have been shown to suppress NF-κB activation in other scenarios.[32] We therefore hypothesized that pioglitazone enhances FC-induced apoptosis at least in part through inhibition of FC-induced NF-κB. In support of this hypothesis, pioglitazone markedly suppressed FC-induced nuclear translocation of NF-κB p65, a measure of NF-κB activation, but not total cellular p65 (Figure 4A). As predicted by the nuclear p65 data, pioglitazone treatment reduced mRNA of the NF-κB–dependent gene tumor necrosis factor-α (Figure 4A). Although the mechanism of suppressed nuclear p65 remains to be determined, we found that it was not associated with either reduced Iκ kinase activity or increased expression of the NF-κB inhibitor Iκ Bα (data not shown). Importantly, when the cells were treated with the Iκ kinase β inhibitor PS-1145, which effectively suppresses NF-κB activa-

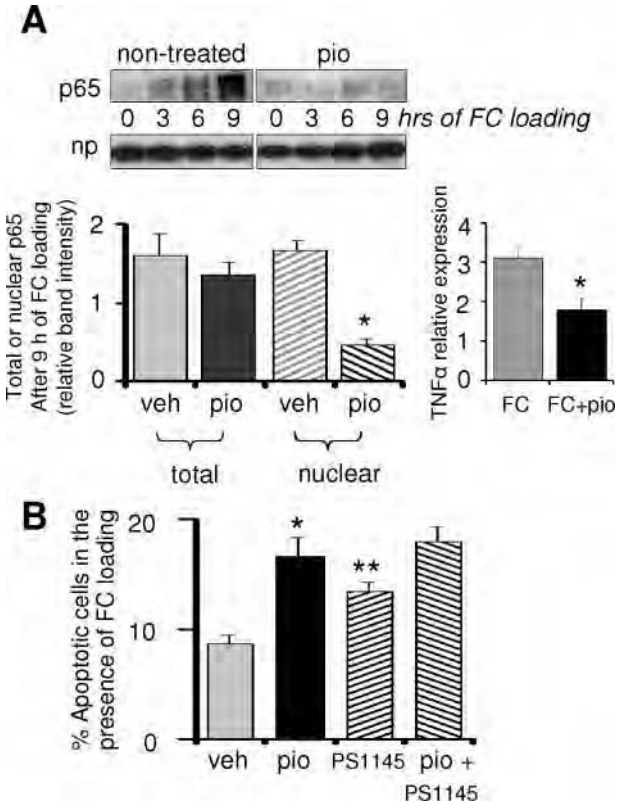

Figure 4. Pioglitazone suppresses nuclear NF-κB-p65, a cell-survival factor, in FC-loaded macrophages. A, Peritoneal macrophages were incubated with 10 μm pioglitazone (pio) or vehicle control (veh); then, nuclear extracts from these cells were immunoblotted with anti-p65 at the indicated times after FC loading. Blots were stripped and reprobed for nucleophosmin (np) as a nuclear extract protein loading control. The bar graph on the left shows quantification of total and nuclear p65 (normalized to tubulin and nucleophosmin, respectively) in macrophages treated with vehicle alone or pioglitazone and then FC loaded for 9 hours. The bar graph on the right shows the induction of tumor necrosis factor-α mRNA by FC loading with or without pioglitazone. The data are derived from quantitative polymerase chain reaction measurements of tumor necrosis factor-α mRNA relative to 36B4 mRNA. B, Macrophages were pretreated for 18 hours with vehicle control (veh), 10 μmol/L pioglitazone, 10 μmol/L PS-1145, or pioglitazone plus PS-1145. The cells were then loaded with FC (with or without the same reagents) for 11 hours and assayed for apoptosis. *$P<0.05$ vs vehicle control.

tion in FC-loaded macrophages,[30,33] enhancement of FC-induced apoptosis was similar to that seen with pioglitazone (Figure 4B). Coincubation of pioglitazone with PS-1145 did not cause an additive increase of apoptosis, consistent with a similar proapoptotic mechanism for NF-κB activation and pioglitazone treatment. Moreover, pioglitazone treatment did not inhibit the expression of a number of prosurvival molecules, including phospho-Akt, Bcl-2, and apoptosis inhibitor of macrophages in FC-loaded macrophages. These data suggest that the suppression of NF-κB contributes to the enhancement of apoptosis by TZDs in wild-type macrophages, but other mechanisms are likely involved (see Discussion section).

TZDs Enhance Efferocytosis of Apoptotic Macrophages

Postapoptotic necrosis of apoptotic macrophages, resulting from inefficient efferocytosis of these cells by neighboring macrophage phagocytes, is thought to be an important contributor to advanced lesional plaque necrosis.[17] To determine the effect of TZDs on efferocytosis, we first treated monolayers of red fluorescently labeled macrophages ("phagocytes") with or without pioglitazone or rosiglitazone. Green fluorescently labeled FC-induced apoptotic macrophages (FC-AMs) were then added to these phagocytes, and efferocytosis was assayed and quantified as described in Methods. As shown in Figure 5A and 5B, TZD-treated phagocytes ingested significantly more FC-AMs, as indicated by the number of red phagocytes stained with green

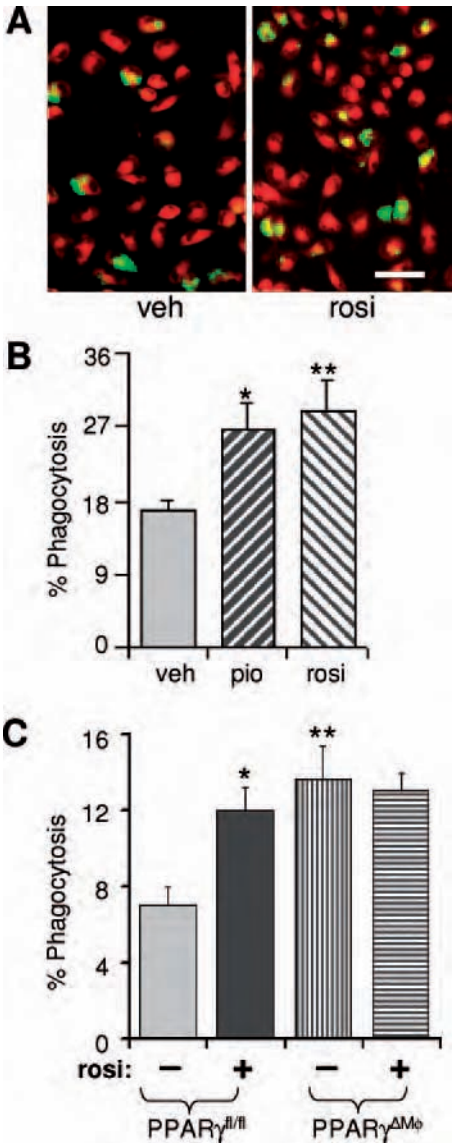

Figure 5. Efferocytosis of apoptotic macrophages is enhanced by TZDs and by PPARγ deficiency. A, Monolayers of red fluorescently labeled macrophages ("phagocytes") were treated for 18 hours with vehicle (veh) control or 1 μmol/L rosiglitazone. FC-AMs were then added to these phagocytes for 30 minutes. After vigorous rinsing to get rid of noninternalized FC-AMs,[23] the monolayers were viewed by fluorescence microscopy. B, Quantification of efferocytosis in phagocytes treated with vehicle control, 10 μmol/L pioglitazone, or 1 μmol/L rosiglitazone. *$P<0.05$ vs vehicle control. C, Peritoneal macrophages from PPARγ$^{fl/fl}$ or PPARγ$^{\Delta M\varphi}$ mice were treated with vehicle control or 1 μmol/L rosiglitazone and then overlaid with FC-AMs and quantified for efferocytosis. *$P<0.05$ vs PPARγ$^{fl/fl}$ phagocytes.

FC-AMs. TZDs also enhanced the efferocytosis of macrophages rendered apoptotic by ultraviolet treatment (data not shown). Enhancement of phagocytosis by TZDs was specific to apoptotic cells because there was no effect on phagocytosis of immunoglobulin-opsonized sheep erythrocytes (data not shown).

To determine the effect of phagocyte PPARγ depletion on efferocytosis, we compared efferocytosis by phagocytes from $PPAR\gamma^{fl/fl}$ and $PPAR\gamma^{\Delta M\varphi}$ mice. A comparison of the first and third bars in Figure 5C revealed an unexpected result, namely that PPARγ depletion was associated with enhanced efferocytosis even in the absence of pioglitazone. This finding suggests that basal expression of PPARγ in macrophage phagocytes suppresses efferocytosis or that a secondary compensatory response to PPARγ depletion in macrophages triggers a pathway that enhances efferocytosis. On the other hand, TZD treatment of PPARγ-deficient phagocytes caused no further enhancement of efferocytosis (compare the third and fourth bars in Figure 5C). Although reconciling these data into a coherent mechanism requires further investigation, the data may suggest opposing effects of PPARγ expression per se versus TZD-mediated activation of PPARγ on efferocytosis.

Pioglitazone Increases Plaque Necrosis in Advanced Atherosclerotic Lesions of LDL Receptor–Deficient Mice

The enhancement of apoptosis by TZDs, in the setting of advanced lesions, would be predicted to promote plaque necrosis, whereas the enhancement of efferocytosis would be predicted to lessen plaque necrosis.[17] To determine the net effect in vivo in a setting in which the insulin-sensitizing effects of TZDs would be minimal, we chose to examine pioglitazone-treated $Ldlr^{-/-}$ mice fed a nondiabetogenic, semisynthetic, low-fat, high-cholesterol diet.[25] Importantly, the mice were administered pioglitazone only after midstage lesions had already developed so that the focus would be on the effect of the drug on advanced lesion progression. Specifically, 6-week-old $Ldlr^{-/-}$ mice were fed the cholesterol-rich diet for 8 weeks, and then the semisynthetic diet was continued for 10 additional weeks in the presence or absence of pioglitazone. We subsequently assessed plasma metabolic and lipid parameters and performed morphometric lesion analysis at the aortic root. The mice were moderately hyperinsulinemic, and although there was a trend toward lower insulin levels in the pioglitazone-treated group, the difference did not reach statistical significance (Figure 6A). There was no hyperglycemia in either group. The pioglitazone-treated mice had ≈10% decrease in plasma total cholesterol and ≈25% increase in high-density lipoprotein (HDL) cholesterol. Fast-performance liquid chromatography of plasma lipoproteins showed that the pioglitazone-treated mice had cholesterol reductions in very LDL and LDL fractions (Figure 6B).

Analysis of plaque morphology revealed substantial differences between the control and pioglitazone-treated groups. As illustrated by the trichrome- and hematoxylin and eosin–stained images in Figure 7A and the quantified data in Figure 7B, plaques from the pioglitazone-treated mice had substantially less collagen content and an increase in areas that were anuclear, afibrotic, and eosin negative. Immunohistochemistry showed the presence of macrophage debris in these regions (data not shown), consistent with "necrosis." Elastin staining in plaques was similar between the 2 groups (data not shown). Importantly, total lesion area per se was not different between the 2 groups of mice (Figure 7C), so the differences in plaque morphology cannot be ascribed simply to differences in overall plaque size. Finally, nuclear-specific TUNEL staining revealed an increase in macrophage apoptosis in the lesions of pioglitazone-treated mice, which occurred in the macrophage-rich areas of these lesions (Figure 8). Pioglitazone treatment did not increase the number of TUNEL-positive macrophages in nonatherosclerotic tissues, eg, in lung and spleen (data not shown). In summary, nondiabetic $Ldlr^{-/-}$ mice treated with pioglitazone after prior development of midstage lesions showed a marked decrease in plaque collagen and a substantial increase in plaque necrosis and macrophage apoptosis. These morphometric parameters, which indicate increased plaque progression in the pioglitazone-treated group, occurred despite a slight improvement in metabolic parameters by the drug and no difference in overall lesion area per se.

A

	non-treated	pioglitazone	p
Body Weight (g)	32.7 ± 0.5	32.9 ± 0.6	0.78
Glucose (mg/dl)	76.1 ± 2.5	81.2 ± 2.0	0.12
Insulin (pg/ml)	1,513.6 ± 106.4	1,335 ± 131.5	0.09
Total Cholesterol (mg/dl)	1,138.2 ± 33.9	949.8 ± 39.3	<0.01
HDL-Cholesterol (mg/dl)	64.5 ± 3.9	90.7 ± 4.5	<0.01
Triglycerides (mg/dl)	130.4 ± 12.7	109.7 ± 15.9	0.32

B

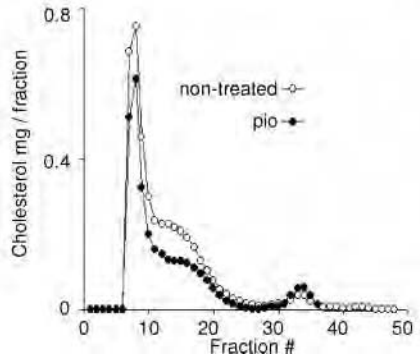

Figure 6. Metabolic parameters of control and pioglitazone (pio)-treated, cholesterol-fed $Ldlr^{-/-}$ mice. $Ldlr^{-/-}$ mice were placed on a semisynthetic, cholesterol-containing diet at 6 weeks of age. After 8 weeks on this diet, the mice were continued for 10 additional weeks on the same diet alone (control) or on a diet containing pioglitazone. A, Plasma from the mice was analyzed for the indicated metabolic parameters (n=25 for control, n=26 for pioglitazone). B, Pooled plasma samples from each group of mice were subjected to fast-performance liquid gel-filtration chromatography.

Discussion

The insulin-sensitizing effects of TZDs would be expected to lessen the incidence of atherothrombotic macrovascular dis-

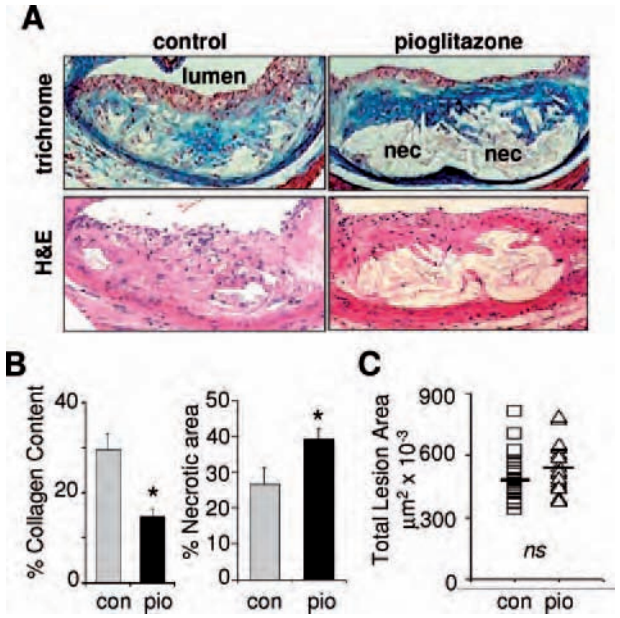

Figure 7. Total lesion area, necrosis, and collagen content in aortic roots from pioglitazone-treated, cholesterol-fed *Ldlr*$^{-/-}$ mice. A, Representative sections of aortic roots from control (con) and pioglitazone-treated (pio) mice were stained with Masson's trichrome stain and with hematoxylin and eosin. Collagen stains blue and cytoplasm stains red in the trichrome method. B, Quantification of percent collagen content and percent anuclear, afibrotic, and eosin-negative (necrotic) area per total plaque area (n=20 plaques for control, n=20 plaques for pioglitazone). C, Quantitative analysis of atherosclerotic lesion area (n=25 for control, n=26 for pioglitazone). Nec indicates plaque necrosis; ns, not significant. *$P<0.05$.

ease in subjects with type II diabetes. Indeed, although the benefit of pioglitazone in macrovascular disease in diabetics was not statistically significant in the PROactive trial using a composite primary end point, analysis of important individual end points suggested a beneficial effect both in this study and in a recently published meta-analysis.[10,12] However, as with any drug, there are likely to be multiple effects. Some of the adverse effects of rosiglitazone on coronary artery disease probably reflect specific effects of this 1 compound.[11,12] In that sense, it was extremely fortunate that the present plaque necrosis study used pioglitazone and not rosiglitazone, because a study showing that rosiglitazone increased plaque necrosis could have simply reflected the specific adverse effect of that 1 compound, with no relevance to TZDs that are likely to be used in the future. However, even in the case of pioglitazone, the overall beneficial effect on acute coronary syndromes in diabetics may reflect a balance between protective mechanisms (eg, insulin sensitization and antiinflammatory processes) and adverse processes that promote plaque necrosis. It was in this context that we sought to explore the effects of TZDs in the following 2 settings: specific cellular events thought to be associated with plaque progression and an in vivo model that emphasizes effects on advanced plaque progression while de-emphasizing effects on either early atherogenesis or the insulin-sensitizing effects of TZDs.

The key finding was the plaque morphology data in the *Ldlr*$^{-/-}$ mouse study. Pioglitazone, when administered to mice with pre-established lesions, resulted in plaques that had signs of increased necrosis, decreased collagen content, and increased macrophage apoptosis despite lower plasma total cholesterol, increased HDL cholesterol, and unaltered overall lesion area. The number of apoptotic macrophages in the lesions of pioglitazone-treated mice was a relatively small percentage of total lesional macrophages, but these levels are consistent with previous studies in which increased apoptosis was associated with increased plaque necrosis.[34] Moreover, the apoptotic cells were found mostly near the edges of expanding necrotic cores. Note that TUNEL staining reflects the number of apoptotic cells at 1 point in time, whereas plaque necrosis likely results from the gradual accumulation over a much longer period of time of apoptotic macrophages that become secondarily necrotic as a result of failure of phagocytic clearance.[17] Overall, the in vivo data in this report are consistent with the conclusion that pioglitazone can promote advanced plaque progression in a model in which the beneficial insulin-sensitizing effects do not come into play.

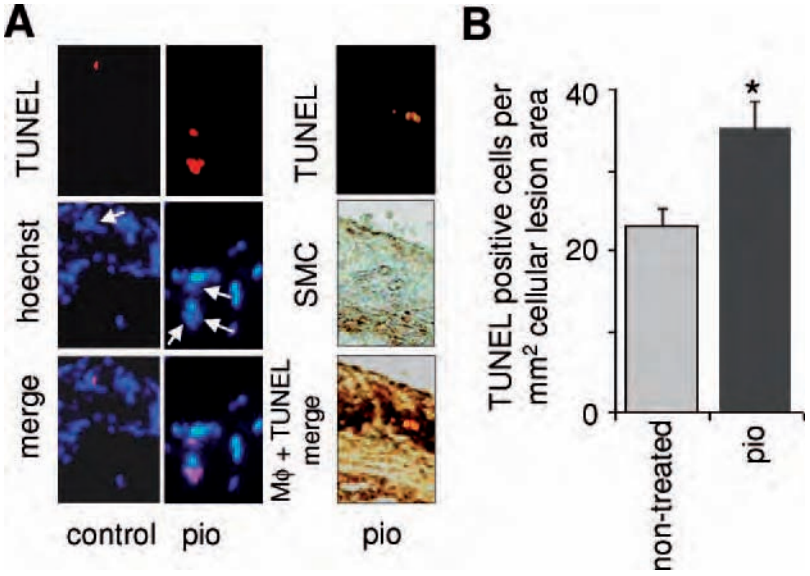

Figure 8. Macrophage apoptosis is increased in advanced aortic root lesions of pioglitazone-treated, cholesterol-fed *Ldlr*$^{-/-}$ mice. A, Representative images show TUNEL-positive cells (red) in sections of aortic root lesions from control and pioglitazone-treated, cholesterol-fed *Ldlr*$^{-/-}$ mice; the sections also were stained with Hoechst nuclear dye (blue). Also shown are sections of an aortic root lesion from pioglitazone-treated mice that were stained for TUNEL (top), smooth muscle cells (middle), and macrophages (Mφs)+TUNEL (bottom). B, Quantification of nuclear-specific TUNEL data (n=25 for control, n=26 for pioglitazone). *$P<0.05$.

Two points relative to our cellular mechanistic studies deserve comment. First, Chinetti et al[35] reported that TZDs induce apoptosis of nonactivated differentiated macrophages in vitro. In our hands, pioglitazone did not induce apoptosis de novo but rather enhanced cell death in response to apoptosis inducers such as FC enrichment of macrophages. This is an important distinction because cholesteryl ester–rich foam cells predominate in early lesions, whereas FC-loaded macrophages are a feature of advanced lesions.[19] The lack of de novo apoptosis induction by TZDs in cholesteryl ester–loaded macrophages is consistent with our finding of no increase in macrophage apoptosis in aortic root lesions from a small group (n=6) of mice treated with pioglitazone during early lesion development (data not shown). Second, our data suggest that suppression of NF-κB participates in the enhancement of apoptosis by TZDs in wild-type macrophages, but the mechanism is undoubtedly more complex. For example, the fact that non-TZD PPARγ agonists do not enhance apoptosis (Figure 3A) but probably still suppress NF-κB[32] raises the possibility that TZDs have additional proapoptotic mechanisms not shared with non-TZD PPARγ agonists or that non-TZDs actively promote cell-survival signaling in a manner that counteracts the suppression of NF-κB. Moreover, we found that pioglitazone did not suppress NF-κB in PPARγ-deficient macrophages (data not shown), despite being able to enhance apoptosis in these cells (Figure 3B). These data suggest that in the special case of PPARγ-deficient macrophages, a mechanism other than suppression of NF-κB is involved in the enhancement of apoptosis by TZDs. This alternative proapoptotic mechanism may represent some sort of "compensatory" response to the chronic absence of PPARγ in these cells. Future mechanistic studies are required to sort out these additional complexities.

Our cell culture studies also showed an enhancing effect of TZDs on efferocytosis of apoptotic macrophages. From a number of studies, this effect, if translated in vivo, might be expected to lessen plaque necrosis.[17] The fact that the overall in vivo effect of pioglitazone was increased, not decreased, plaque necrosis may indicate that this action of pioglitazone does not occur in the setting of advanced atherosclerosis or that other plaque-promoting effects of pioglitazone such as enhancement of macrophage death play a dominant role. Nonetheless, pending further mechanistic studies, future drug refinement may be able to take advantage of this potentially beneficial effect of TZDs.

In summary, the data in the present report reveal an action of TZDs that promotes advanced plaque progression in *Ldlr*$^{-/-}$ mice through a mechanism that may involve enhancement of advanced lesional macrophage apoptosis. Key future goals are to determine whether TZDs promote advanced lesional macrophage apoptosis and plaque necrosis in PPARγ-deficient *Ldlr*$^{-/-}$ mice, as predicted, and to further probe cellular and molecular mechanisms of TZD and PPARγ effects on apoptosis and efferocytosis. Our overall contention is that PPARγ-dependent improvement in insulin resistance has the potential to decrease cardiovascular disease in diabetic patients but that this benefit will be optimally realized only if the potential detrimental effects of even "good" TZDs such as pioglitazone be eliminated through drug optimization.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Thiazolidinediones (TZDs) are widely prescribed drugs that improve insulin sensitivity in patients with type II diabetes mellitus. Because insulin resistance is linked to accelerated atherogenesis, TZDs have the potential to decrease the incidence of atherothrombotic vascular disease. Despite evidence that TZDs reduce early atherosclerotic lesions in mice, their effects on advanced atherosclerosis, which is more relevant to acute coronary syndromes in humans, are not known. On the one hand, the Prospective Pioglitazone Clinical Trial in Macrovascular Events failed to show a beneficial effect of pioglitazone in diabetic patients on the primary composite end point of all-cause mortality, nonfatal myocardial infarction, stroke, acute coronary syndrome, and peripheral vascular disease. Moreover, a recent meta-analysis by Nissen's group has linked the TZD rosiglitazone with a greater risk of heart attack. On the other hand, pioglitazone was found to have an overall beneficial effect in diabetic patients on the individual end points of all-cause mortality, nonfatal myocardial infarction, and stroke in both the Prospective Pioglitazone Clinical Trial in Macrovascular Events and in a recent second meta-analysis by Nissen and colleagues. How can one reconcile these findings? The current study in nondiabetic mice showing that pioglitazone accelerates advanced plaque development and that the mechanism appears to be independent of peroxisome proliferator–activated receptor-γ suggests a possible explanation. First, Nissen's 2 recent meta-analyses suggest that rosiglitazone has adverse cardiovascular effects that are probably specific to this 1 compound. In that sense, it was extremely fortunate that the current plaque necrosis study used pioglitazone and not rosiglitazone, because a study showing that rosiglitazone increased plaque necrosis could have simply reflected the specific adverse effect of that 1 compound, with no relevance to TZDs that are likely to be used in the future. In terms of pioglitazone, the current plaque necrosis study raises the possibility that the overall beneficial effect of pioglitazone reflects a balance between protective mechanisms (eg, insulin sensitization and antiinflammatory processes) and adverse processes that promote advanced plaque necrosis. Moreover, the adverse effects may be due to non–peroxisome proliferator–activated receptor-γ off-target effects of the drug. Therefore, the 'good' TZDs like pioglitazone may be made even better if their potential detrimental effects are eliminated through drug optimization. Whether such optimization could also eliminate the more widely observed adverse effects of TZDs on heart failure remains to be seen.

STAT1 is Critical for Apoptosis in Macrophages Subjected to Endoplasmic Reticulum Stress *in Vitro* and in Advanced Atherosclerotic Lesions *in Vivo*

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Running Title: STAT1 and Macrophage Death in Atherosclerosis

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Abstract

Background—Macrophage apoptosis is a critical process in the formation of necrotic cores in vulnerable atherosclerotic plaques. *In-vitro* and *in-vivo* data suggest that macrophage apoptosis in advanced atheromata may be triggered by a combination of endoplasmic reticulum (ER) stress and engagement of the type A scavenger receptor (SRA), which together induce death through a rise in cytosolic calcium and activation of toll-like receptor-4 (TLR4).

Methods and Results—Using both primary peritoneal macrophages and studies in advanced atheromata *in vivo*, we introduce Signal Transducer and Activator of Transcription-1 (STAT1) as a critical and necessary component of ER stress/SRA-induced macrophage apoptosis. We show that STAT1 is serine-phosphorylated in macrophages subjected to SRA ligands and ER stress in a manner requiring cytosolic calcium, calcium/calmodulin-dependent protein kinase II (CaMKII), and TLR4. Remarkably, apoptosis was inhibited by ~80-90% ($p < 0.05$) by STAT1 deficiency or CaMKII inhibition. *In vivo*, nuclear Ser-P-STAT1 was found in macrophage-rich regions of advanced murine and human atheromata. Most importantly, macrophage apoptosis was decreased by 61% ($p = 0.034$) and plaque necrosis by 34% ($p = 0.02$) in the plaques of fat-fed *Ldlr*-/- mice transplanted with *Stat1*-/- bone marrow.

Conclusions—STAT1 is critical for ER stress/SRA-induced apoptosis in primary tissue macrophages and in macrophage apoptosis in advanced atheromata. These findings suggest a potentially important role for STAT1-mediated macrophage apoptosis in atherosclerotic plaque progression.

Key Words: atherosclerosis • plaque necrosis • macrophage • apoptosis • STAT1

In advanced atherosclerosis, death of macrophages in the setting of defective phagocytic clearance of apoptotic cells contributes to the development of plaque necrosis.[1,2] Plaque necrosis, in turn, is thought to promote plaque disruption and arterial thrombosis, which are the proximate causes of acute cardiovascular events.[1-3] Our laboratory established an important principle of advanced lesional macrophage death, namely, involvement of the ER stress pathway known as the Unfolded Protein Response (UPR).[4,5] The laboratories of Austin, Lusis, and others have important evidence that the UPR is activated in intimal cells, including macrophages, in advanced murine and human plaques.[6-9] In particular, Myoishi *et al*.[9] recently showed a dramatic rise in UPR markers, including CHOP, and intimal cell apoptosis in autopsy specimens from humans with vulnerable and ruptured plaques, but not stable lesions, and in atherectomy specimens from humans with unstable angina, but not stable angina. Although the UPR is primarily an ER repair pathway, a branch of the UPR involving the effector CHOP (GADD153) can trigger apoptosis when the cell senses that repair is no longer possible.[4,10] In terms of causation, we have shown that advanced lesional macrophage death and plaque necrosis are decreased in atherosclerotic *Apoe*-/- mice in the setting of ER stress prevention[5] or CHOP deficiency (E. Thorp and I. Tabas, unpublished data).

Our work on the UPR began with a model of advanced lesional macrophage death that is present in advanced plaques, namely, intracellular accumulation of lipoprotein-derived free cholesterol (FC).[11] FC enrichment of macrophages, like many ER stressors, activates the UPR through depletion of ER lumenal calcium.[12,13] Since then, mechanistic studies have led to a broader concept of advanced lesional macrophage death, beyond the FC model. These studies have shown that any combination of inducers of ER stress and ligands for the macrophage type A scavenger receptor (SRA), both of which are prominently expressed in advanced lesions, trigger macrophage apoptosis.[14,15] Macrophage SRA recognizes a number of lesional molecules and atherogenic lipoproteins, including those used to enrich macrophages with cholesterol in the FC model.[16] The SRA is also a pattern recognition receptor (PRR) of the innate immune system, and endotoxin-free SRA ligands also activate other PRRs, notably TLR4.[15,17,18] In this context, our studies have shown that SRA ligands trigger two critical pro-apoptotic events in ER-stressed macrophages: **(a)** TLR4-mediated activation of a pro-apoptotic MyD88 pathway;[15] and **(b)** SRA-mediated suppression of a pro-survival TLR4-TRIF-INFβ pathway.[14,15]

In this report, we show that apoptosis of ER-stressed macrophages also requires STAT1 and CaMKII in a process involving cytosolic calcium and TLR4. Most importantly, we provide evidence that activated STAT1 is present in atheromata and that lesional macrophage apoptosis is suppressed in the setting of STAT1 deficiency.

Methods

(See Data Supplement for expanded methods)

Assay of Macrophage Apoptosis

Mid- and late-stage apoptosis in peritoneal macrophages was assayed by annexin V and propidium iodine (PI) staining, respectively, using the Vybrant Apoptosis Assay kit #2 (Molecular Probes). At the end of incubation, the macrophages were gently washed once with PBS and incubated for 15 min at room temperature with 120 μl of annexin-binding buffer (25 mM HEPES, 140 mM NaCl, 1 mM EDTA, pH 7.4, 0.1% bovine serum albumin) containing 10 μl of Alexa Fluor 488-conjugated annexin V and 1 μl of 100 μg/ml PI. The staining mixture was then removed and replaced with 120 μl of annexin-binding buffer. The cells were viewed immediately at room temperature using an Olympus IX-70 inverted fluorescent microscope equipped with filters appropriate for fluorescein and rhodamine, and images were obtained using a Cool Snap CCD camera (RS Photometrics) equipped with imaging software from Roper Scientific. Three fields of cells (~650 cells/field) were photographed for each condition, and the number of annexin V/PI–positive cells in each field were counted and expressed as a percent of the total number of cells.

Bone Marrow Transplantation

Ten-week-old female *Ldlr*-/- mice were lethally irradiated with 1000 rads from a cesium γ source 4-6 h before transplantation. Bone marrow cells were collected from the femurs and tibias of donor *Stat1*-/- or *Stat1*+/+ mice by flushing with sterile medium (RPMI 1640, 2% FBS, 10 U/ml heparin, 50 U/ml penicillin, 50 μg/ml streptomycin). The bone marrow cells were extensively washed and resuspended in RPMI medium containing 20 mM HEPES, 50 U/ml penicillin, and 50 μg/ml streptomycin. Each recipient mouse was injected with 5×10^6 bone marrow cells through the tail vein. The mice were given acidified water containing 100 mg/liter neomycin and 10 mg/liter polymyxin B sulfate 1 week before and 2 weeks after transplantation. Six weeks

after transplantation, the mice were fed a "Western-type" diet (21% anhydrous milk fat and 0.15% cholesterol—TD88137 from Harlan-Teklad) for 10 or 12 weeks.

Atherosclerotic Lesion Analysis

On the day of the analysis, food was removed from the cages in the morning, and the mice were fasted for 8 h. The animals were then anesthetized using isoflurane, and blood was withdrawn by cardiac puncture. The heart was then perfused with PBS, and the heart and proximal aorta were harvested. The heart and aorta were perfused *ex vivo* with PBS and then transferred to 10% buffered formalin, processed, and embedded in paraffin. Starting from the atrial leaflets, serial sections (6-μm thick) were prepared such that every eighth section was stained with Harris hematoxylin and eosin. Atherosclerotic lesions in six sections were analyzed in a blinded fashion using a Nikon Labophot-2 microscope equipped with a Sony CCD-Iris/RGB color video camera attached to a computerized imaging system using IMAGE-PRO PLUS 3.0 software. Aortic lesion area was quantified by averaging the lesion areas of the six sections. Necrotic areas were defined as those regions of the lesions that lacked nuclei and cytoplasm.

***In-situ* TUNEL Assays**

Apoptotic cells in the intima of atherosclerotic lesions were detected by the TUNEL (TdT-mediated dUTP nick-end labeling) technique using the TMR red *in-situ* cell death detection kit (Roche) and the stringent method of Kockx.[19] Sections of proximal aorta were deparaffinized, rehydrated, and treated with 2 μg/ml Proteinase K (Roche) for 30 min at 37°C in a humidified chamber. The treated sections were incubated in TdT reaction mixture containing TMR red dUTP for 1 hr at 37°C in a humidified chamber. After washing, genomic DNA was stained with DAPI for 5 min at room temperature, and then the slides were mounted with coverslips. TUNEL staining was analyzed using an Olympus IX-70 inverted fluorescent microscope equipped with a Cool Snap CCD camera and imaging software (Roper Scientific). Fluorescent images were captured and analyzed using image analysis software Photoshop (Adobe).

Statistics

Data are presented as mean ± S.E.M.. Absent error bars in the bar graphs signify S.E.M. values smaller than the graphic symbols. Significance of paired data was determined by the Student's *t*

test. Data with >2 groups or ≥2 independent variables were analyzed with ANOVA followed by the Bonferroni post hoc test. Significance is indicated by an *asterisk* in the figures, with an explanation in the figure legends, while non-significance is indicated by *ns* in the figures.

Statement of Responsibility

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the mansucript as written.

Results

SRA-Induced Apoptosis in ER-Stressed Macrophages Requires STAT1 and is Preceded by Serine Phosphorylation of STAT1

During the course of another study investigating an ER stress response mediator called PKR, we conceived the hypothesis that STAT1, whose activity is modulated by PKR[20], may play a role in ER stressed-induced macrophage apoptosis. To test this idea, we compared SRA/ER stress-induced apoptosis in peritoneal macrophages from wild-type *vs*. *Stat1*-/- mice. Confirming our previous work, both intracellular FC enrichment with an SRA-interacting lipoprotein and treatment with the SRA ligand fucoidan plus the UPR activator thapsigargin triggered apoptosis, as indicated by an increase in annexin V staining (**Figure 1A-B, *WT***). In contrast, *Stat1-/-* macrophages were markedly protected from apoptosis by both inducers (80-90% inhibition of apoptosis, $p < 0.05$), indicating an essential role of STAT1 in this model of macrophage apoptosis (**Figure 1A-B, *Stat1-/-***). The decrease in apoptosis in *Stat1*-/- macrophages could not be explained by a decrease in either SRA (not displayed) or CHOP induction (**Figure 1C**).

STAT1 is activated by phosphorylation of Y701 or S727.[21] Y701 phosphorylation is essential for STAT1 dimerization, nuclear translocation, and DNA binding.[21] S727 phosphorylation enhances the transcriptional activity of tyrosine-phosphorylated STAT1 or, in some cases, has been reported to participate in signaling in the absence of Y701 phosphorylation.[21-23] As shown in **Figure 2A**, FC loading of macrophages induced serine, whereas tyrosine phosphorylation was not detected, and total STAT1 was not increased. In contrast, very little serine phosphorylation was seen in non-loaded or cholesteryl ester-loaded macrophages, which show no or very little evidence of ER stress.[4] As expected, IFNγ induced highly detectable levels of tyrosine phosphorylation as well as serine phosphorylation of STAT1.[21] Previous work has suggested

that nuclear Ser-P-STAT1 can occur through serine phosphorylation of a constitutive pool of nuclear STAT1.[23] We detected STAT1 in nuclear fractions isolated from untreated macrophages, and Ser-P-STAT1 was increased with FC loading. Although total nuclear STAT1 was modestly increased after FC loading, this increase was much less than that seen with IFNγ, which is known to induce STAT1 nuclear translocation[21] (**Figure 2B**). These data suggest that at least a portion of FC-induced Ser-P-STAT1 occurs through phosphorylation of constitutively nuclear STAT1. It is also possible that at least a portion of the STAT1 was tyrosine phosphorylated but below the limits of detection of our immunoblot assay.

The ability of IFNγ to stimulate both serine and tyrosine phosphorylation of STAT1, the presence of IFNγ in atherosclerotic lesions, and recent evidence that IFNγ promotes advanced plaque progression[24,25] led us to explore the effect of the combination of FC loading and IFNγ treatment on Ser-P-STAT1 and apoptosis. FC-loaded macrophages treated with IFNγ showed an increase in Ser-P-STAT1 that was greater than either condition alone (**Figure 3A**). Note that IFNγ alone did not induce CHOP, nor did it further increase CHOP in the setting of FC loading. Most importantly, under conditions in which IFNγ alone induced no apoptosis, IFNγ treatment led to a > 5-fold enhancement of FC-induced apoptosis (**Figure 3B, *WT***). This effect of IFNγ required STAT1, because it was inhibited by 93% ($p < 0.05$) in *Stat1-/-* macrophages (**Figure 3B, *Stat1-/-***). Thus, in atheromata, where macrophages are likely exposed to the combination of SRA ligands, ER stressors, and IFNγ, the role of STAT1 in macrophage apoptosis may be particularly important (below).

Cytosolic Calcium, TLR4, and CaMKII Activation are Required for Stat1 Serine Phosphorylation and Apoptosis in FC-Loaded Macrophages

Three kinases that are able to catalyze serine phosphorylation of STAT1 are p38, ERK, and protein kinase C-δ.[23] However, using a combination of gene targeting and chemical inhibitors, we found that inhibition of these kinases did not abrogate FC-induced serine phosphorylation of STAT1 (data not shown). In the face of these negative data, we next asked whether two critical components of the multi-hit model, ER stress and TLR4 signaling, were necessary for STAT1 serine phosphorylation. The data in **Figure 4A-B** show that blocking FC-induced ER stress by the cholesterol trafficking inhibitor U18666A[4] or omitting thapsigargin from the fucoidan-plus-

thapsigargin model markedly suppressed Ser-P-STAT1. In addition, FC-induced serine phosphorylation of STAT1 was almost completely prevented in TLR4-deficient macrophages (**Figure 4C**). Note that all of these manipulations also block macrophage apoptosis.[4,15]

Both ER stress and TLR4 signaling can affect cellular calcium metabolism (see Discussion).[12,13,15,26,27] Moreover, we recently showed that buffering cytosolic calcium with BAPTA-AM markedly inhibited both FC-induced and thapsigargin/fucoidan-induced apoptosis.[15] To test the role of cytosolic calcium in STAT1 serine phosphorylation, we incubated FC-loaded macrophages with increasing concentrations of BAPTA-AM or equivalent volumes of vehicle control. As shown in **Figure 5A**, BAPTA-AM suppressed FC-induced serine phosphorylation of STAT1 in a dose-dependent manner.

One mechanism by which cytosolic calcium might participate in STAT1 serine phosphorylation is by activating CaMKII, which may directly phosphorylate STAT1[28] and/or lead to its phosphorylation through enhancing TLR4 signaling (see Discussion).[15,29,30] As shown in **Figure 5B**, FC loading led to a rapid and marked enhancement of CaMKII threonine phosphorylation, which is a marker of its activation. At the 30- and 60-min timepoints, the degree of activation was similar to that of the calcium ionophore A23187, a known potent activator of CaMKII. Similar results were found with fucoidan plus thapsigargin (data not shown). Note that the time course of CaMKII activation by FC loading or by thapsigargin plus fucoidan precedes the onset of STAT1 serine phosphorylation in these cells. To show a functional role for CaMKII activation in both STAT1 serine phosphorylation and apoptosis in FC-loaded macrophages, we utilized two structurally diverse CaMKII inhibitors. The data in **Figure 5C-D** show that the chemical CaMKII inhibitor KN93[31], but not the inactive homologue KN92, and the peptide CaMKII inhibitor AIP[32] markedly suppressed FC-induced STAT1 serine phosphorylation. Most importantly, KN93, but not KN92, suppressed FC-induced apoptosis by 92% ($p < 0.05$) (**Figure 5E**). Note that neither KN93 nor AIP decreased the uptake or ER-trafficking of lipoprotein-derived FC or the induction of CHOP (data not shown and Figure 5D). In summary, these data indicate that cytosolic calcium and CaMKII are essential for STAT1 serine phosphorylation and apoptosis in the SRA-ER stress model. We also conducted experiments on two additional macrophage models, namely, mouse bone marrow-derived and human peripheral blood-derived macrophages. In both of these cell types, the SRA-ER stress model exclusively induced STAT1 serine phosphorylation via a pathway mediated by cytosolic

calcium and CaMKII (***see Figure in the online Data Supplement***), suggesting the universality of this signaling pathway among macrophages.

STAT1 is Serine-Phosphorylated in Murine and Human Atherosclerotic Lesions, and STAT1 Plays a Role in Advanced Lesional Macrophage Apoptosis and Plaque Necrosis in Female *Ldlr*-/- Mice

To provide evidence for the relevance of Ser-P-STAT1 in atherosclerosis, we first used immunohistochemistry to assess expression of Ser-P-STAT1 in murine and human atheromata (**Figure 6 and 7**). In mouse lesions, Ser-P-STAT1 was present in numerous macrophage foam cells, as assessed by staining adjacent sections with anti-Mac-3 antibody (**Figure 6A,B**) and Oil-Red O (**Figure 6D**). As illustrated by these images, Ser-P-STAT1 staining was also observed in the endothelial cells lining the lumen, which was PECAM-1-positive (not shown), and in smooth muscle cells in the media, which were α-actin positive (**Figure 6F**). In human lesions, staining of Ser-P-STAT1 was found in the advanced stages termed pathological intimal thickening and fibroatheroma (**Figure 7B,C**), but not in the early stage of diffuse intimal thickening (**Figure 7A**). In the advanced lesions, most of the Ser-P-STAT1 co-localized with macrophages (**Figure 7B,C**). Note that Ser-P-STAT1 was found in the nuclei of these cells (**Figure 7C, *bottom middle image***) and in areas that were TUNEL-positive, a marker of apoptosis (**Figure 7C, *bottom right image***). Of interest, some of the Ser-P-STAT1 in the most advanced fibroatheroma was found in macrophages surrounding necrotic areas (**Figure 7C, *bottom left image, asterisk***).

To further investigate a causal link between STAT1 and lesional macrophage apoptosis, we compared advanced plaques of Western diet-fed *Ldlr*-/- mice reconstituted with either wild-type or *Stat1*-/- bone marrow. The mice were fed the Western diet for 10 or 12 weeks. Plasma lipoprotein cholesterol and body weight were similar between the two groups of mice (**Figure 8A** for 10-wk protocol; data not shown for 12-wk protocol). In the 10-wk study, overall lesion area was similar (**Figure 8B,C**). However, the number of TUNEL-positive cells in macrophage-rich regions was deceased by 61% ($p = 0.034$) in the *Stat1*-/- $\rightarrow$ *Ldlr*-/- lesions, and there was a trend towards decreased plaque necrosis that did not quite reach statistical significance ($p = 0.078$) (**Figure 8C**). Note that total macrophage area was not affected by STAT1 deficiency ($120.0 \pm 11.8 \times 10^3$ μm^2 and $111.5 \pm 21.3 \times 10^3$ μm^2 in wild-type and *Stat1*-/- bone marrow recipients respectively, $p = 0.72$; see Discussion).

Plaque necrosis likely results from the eventual cellular necrosis of macrophages that become apoptotic but are not subsequently cleared by phagocytes.[1,2] Therefore, we predicted that as the lesions in the two groups of mice progressed, the difference in necrotic core areas would become statistically significant, while apoptotic macrophages *per se* would become less numerous and less different between the two groups of mice. As shown by the data in **Figure 8D**, the necrotic cores were larger in the 12-wk-diet mice, and there was a statistically significant difference in the necrotic core area (34% decrease in the *Stat1*-/- → *Ldlr*-/- lesions, p = 0.02) but not the number of TUNEL-positive cells. In summary, STAT1 deficiency in bone marrow-derived cells in *Ldlr*-/- mice has a substantial protective effect on apoptosis in macrophage-rich lesions of advanced plaques and on plaque necrosis.

Discussion

There is increasing evidence from a number of laboratories that an ER stress-based model of macrophage apoptosis plays an important role in advanced lesional macrophage death and plaque necrosis.[4-9] The work in this report adds critical new components to this model by demonstrating essential roles for STAT1 and CaMKII in macrophage apoptosis *in vitro* and for STAT1 in advanced lesional macrophage apoptosis and plaque necrosis *in vivo*.

Future studies will be required to define at a precise molecular level how the pro-apoptotic components elucidated in this study fit into the overall scheme of the multi-hit model of macrophage apoptosis. Our working hypothesis is depicted in **Figure 9**. We suggest that ER stress triggers two key pro-apoptotic processes: UPR/CHOP and another pathway in which ER stress-induced cytosolic calcium activates CaMKII, which in turn leads to serine phosphorylation of pro-apoptotic STAT1. Activation of the TLR4-MyD88 pathway by SRA ligands, which is critical for apoptosis[15], also contributes to STAT1 serine phosphorylation. SRA ligands additionally promote apoptosis through SRA-dependent suppression of pro-survival IFNβ.[14,15]

This scheme raises a number of critical issues that will require further investigation. Among these is whether STAT1 serine-phosphorylation *per se* is required for apoptosis, which is consistent with our data and with previous work showing a pro-apoptotic role of Ser-P-STAT1 in apoptosis in other systems.[33] However, definitive proof will require comparing SRA/ER stress-induced apoptosis in macrophages containing S727- *vs*. Y701-mutated STAT1.[22,33] Until then,

we cannot definitively rule out the possibility that apoptosis requires Y701 phosphorylation and that Tyr-P-STAT1 in our SRA-ER stress model is below the limit of immunoblot detection. In pilot studies, we found that apoptosis induced by thapsigargin and fucoidan was markedly suppressed in peritoneal macrophages from S727A-STAT1 knock-in mice, but results with FC-induced apoptosis were difficult to interpret due to an as-yet-undefined compensatory pathway (J. Timmins, W. Lim, T. Decker, and I. Tabas, unpublished data). Assuming Ser-P-STAT1 is a key apoptosis mediator in this pathway, the next goal is to elucidate if and how serine-only phosphorylated STAT1 affects gene transcription in a manner that promotes apoptosis.[33-35] A related issue is the precise mechanism by which STAT1 is serine-phosphorylated and how this process is linked to both CaMKII and TLR4 (**Figure 9**). One obvious possibility is that CaMKII directly phosphorylates STAT1, as has been described previously in other models.[28] If this is the case in our model, a link to TLR4 could occur through IP3 receptor-mediated elevation of cytosolic calcium[26], leading to a further increase in CaMKII activation. Alternatively, TLR4 signaling may more directly stimulate STAT1 serine phosphorylation, as has been reported in other models using the TLR4 ligand, LPS.[29,30]

The impetus for this study was to explore pathways that may be involved in promoting macrophage apoptosis in atherosclerosis. The ultimate significance of lesional macrophage apoptosis likely depends on lesion stage.[1,2] In early lesions, rapid and efficient phagocytic clearance of apoptotic macrophages appears to limit lesion cellularity and progression. Of interest, STAT1 may have a separate role in these early lesions independent of macrophage death, because STAT1 deficiency in *Apoe*-/- mice blocks foam cell formation and early lesion development.[36] In advanced lesions, however, there is evidence that clearance of apoptotic cells is defective, leading to post-apoptotic macrophage necrosis, inflammation, and, eventually, overall plaque necrosis.[1,2] In this context, the multi-hit model of macrophage apoptosis is likely most relevant to advanced lesions. For example, immunoblots have shown that CHOP is expressed only in advanced lesions[7], and manipulation of ER stress *in vivo* is positively associated with advanced lesional necrosis, not negatively associated with early lesion progression.[5,37] In the case of STAT1 deficiency, there was a clear trend towards decreased plaque necrosis. However, the maximum effect on plaque necrosis may lag behind that of macrophage apoptosis, because plaque necrosis likely results from the progressive coalescence of apoptotic macrophages after they become secondarily necrotic.[1,2] Another prediction from

this idea and from the fact that the anti-macrophage antibody used in our study recognizes pre-necrotic apoptotic macrophages is that *total* macrophage area should be similar in *Stat1*+/+ → *Ldlr*-/- and *Stat1*-/- → *Ldlr*-/- lesions, exactly as we observed experimentally. More fundamentally, we clearly did not observe an *increase* in lesion area in the *Stat1*-/- group, which is what is found when early lesional macrophage apoptosis is blocked.[38] In terms of other studies linking STAT1 to advanced plaque progression, *in-vivo* data suggest that interleukin-10, which suppresses STAT1 activity[39], may protect advanced atheromata from macrophage apoptosis and plaque necrosis.[40,41] Moreover, Koga *et al*.[25] reported that blocking the function of the STAT1 activator IFNγ stabilized advanced plaques in *Apoe*-/- mice. Thus, pending further *in-vivo* studies, local inhibition of STAT1 activity may represent a potentially promising therapeutic strategy to prevent the progression of relatively benign lesions to those with increased macrophage apoptosis and plaque necrosis.

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Disclosures

None.

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Figure Legends

Figure 1. SRA/ER stress-induced macrophage apoptosis requires STAT1. (***A-B***) Peritoneal macrophages from wild-type (*WT*) or *Stat1-/-* mice were incubated for 17 h with medium alone (*Control*) or medium containing acetyl-LDL plus the ACAT inhibitor 58035 (*FC-loaded*); or 21 h with medium alone (*Control*) of medium containing 50 μg/ml fucoidan and 0.5 μM thapsigargin (*Fuc + Thaps*). Mid- and late-stage apoptosis were assessed by staining with Alexa Fluor 488-conjugated annexin V (*green*) and PI (*orange*), respectively. Representative merged fluorescence and bright-field images and quantitative data from 3 fields of cells for each condition are shown. *, p = 0.001 by Bonferroni after ANOVA. (***C***) Lysates from wild-type and *Stat1-/-* macrophages were FC-loaded for the indicated times and subjected to immunoblot analysis to detect CHOP, total STAT1, and β-actin.

Figure 2. FC loading induces serine- but not tyrosine-phosphorylation of STAT1. (***A***) Macrophages were incubated for the indicated times with medium alone (*Control*) or medium containing acetyl-LDL (*CE-loaded*), acetyl-LDL plus 58035 (*FC-loaded*), or 100 U/ml IFNγ. Whole-cell lysates were then prepared and subjected to immunoblot analysis to detect phospho-S727 STAT1 (*Stat1 pS727*), phospho-Y701 STAT1 (*Stat1 pY701*), and total STAT1. (***B***) Nuclear fractions from control, FC-loaded, and IFNγ-treated macrophages were subjected to immunoblot analysis to detect STAT1 pS727, total STAT1, and the nuclear marker nucleophosmin.

Figure 3. IFNγ enhances FC-induced STAT1 serine phosphorylation and STAT1-dependent FC-induced apoptosis. (***A***) Macrophages were incubated for the indicated times with medium alone (*Control*) or medium containing acetyl-LDL plus 58035 (*FC*), 100U/ml IFNγ, or acetyl-LDL, 58035, and IFNγ (FC + IFNγ). Whole-cell lysates were then prepared and subjected to immunoblot analysis to detect STAT1 pS727, total STAT1, CHOP, and β-actin. In the CHOP blot, a non-specific band is indicated by the *asterisk*. (***B***) Macrophages from wild-type (*WT*) or *Stat1-/-* mice were incubated for 13 h with medium alone (*Control*) or medium containing 100U/ml IFNγ, acetyl-LDL plus 58035 (*FC*), or acetyl-LDL, 58035, and IFNγ (FC + IFNγ).

Apoptosis was assayed and quantified as in Figure 1. *, p = 0.01 for FC and 0.001 for FC + INFγ by Bonferroni after ANOVA.

Figure 4. STAT1 serine phosphorylation in SRA-engaged, ER-stressed macrophages is amplified by ER stress and requires TLR4 activation. Whole-cell lysates were subjected to immunoblot analysis to detect STAT1 pS727 and total STAT1 under the following conditions: (***A***) Macrophages were incubated for the indicated times with acetyl-LDL and 58035 (*FC-loaded*) or acetyl-LDL, 58035, and 1μM U18666A (*FC-loaded + U18666A*). **(*B*)** Macrophages were incubated for the indicated times with 0.5 μM thapsigargin, 50 μg/ml fucoidan plus thapsigargin, or fucoidan alone. (***C***) Macrophages from wild-type (*WT*) or *Tlr4*^{del} mice were incubated for the indicated times under FC-loading conditions.

Figure 5. Cytosolic calcium and CaMKII activation are required for FC-induced STAT1 serine phosphorylation, and inhibition of CaMKII blocks FC-induced apoptosis. (***A-D***), Whole-cell lysates were subjected to immunoblot analysis to detect STAT1 pS727, total STAT1, CaMKII pT286/287, β-actin, or CHOP, as indicated in the individual blots, under the following conditions: (***A***) Macrophages were incubated for 8 h in medium alone (*Control*); medium containing acetyl-LDL and 58035 plus vehicle control (*FC + Vehicle*); or medium containing acetyl-LDL, 58035, and increasing concentrations of BAPTA (*FC + BAPTA*). The indicated microliters of vehicle or BAPTA-AM stock solution (1 mg/ml) were added per ml of medium. (***B***) Macrophages were incubated for the indicated times with medium alone (*Control*) or medium containing acetyl-LDL and 58035 (*FC-loaded*) or 2 μg/ml A23187. (***C***) Macrophages were incubated for the indicated times with acetyl-LDL and 58035 (*FC*) or acetyl-LDL and 58035 plus either 10 μM KN93 or 10 μM KN92 (*FC + KN93* or *FC + KN92*). (***D***) Macrophages were incubated for 8 h with acetyl-LDL and 58035 (*FC*), 10 μM AIP, or acetyl-LDL, 58035, and AIP. (***E***) Macrophages were incubated for 24 h in medium alone (*Control*) or medium containing acetyl-LDL and 58035 (*FC-loaded*) or acetyl-LDL and 58035 plus either 10 μM KN93 or 10 μM KN92 (*FC-loaded + KN93* or *FC-loaded + KN92*). Apoptosis was assayed and quantified as in Figure 1. For all experiments involving KN93, KN92, or AIP, the macrophages were pre-treated for 1 h with medium alone or medium containing these inhibitors prior to FC loading. *, p < 0.01 by Bonferroni after ANOVA.

Figure 6. STAT1 is serine-phosphorylated in atherosclerotic lesions from *Ldlr*-/- mice. Adjacent frozen sections of an aortic root lesion from an *Ldlr*-/- mice fed a Western-type diet for 12 wks were immunostained with anti-Ser-P-STAT1 or anti-Mac3 (macrophages) (***A,B***) or anti-Ser-P-STAT1, Oil Red O, non-immune IgG, and α-actin (***C-F***). Note examples of brown stain in the nuclei of the intimal cells (*red arrows*), endothelial cells (*blue arrows*), and smooth muscle cells in the media (*black arrows*). The dark streaks at the intima-media interface in *panel E* represent non-specific staining.

Figure 7. Ser-P- STAT1 is present in advanced human coronary atheromata but not in diffuse intimal thickening. The sections were stained with Movat pentachrome, anti-Ser-P-STAT1, anti-CD68, and non-immune IgG, as indicated in the images. (***A***) Diffuse intimal thickening. The CD68 and Ser-P-STAT1 images on the right are higher magnifications of the area indicated by the box in the low-magnification Ser-P-STAT1 image. As shown in the higher magnification images, only a few CD68-positive macrophages are present directly under the endothelium (*arrowheads*). Ser-P-STAT1 was not detected. *Arrow* indicates internal elastic lamina (*IEL*). (***B***) Pathological intimal thickening. Ser-P-STAT1 staining coincides with CD68-positive macrophages. (***C***) Fibroatheroma: Ser-P-STAT1 staining coincides with CD68-positive macrophages. Some of the Ser-P-STAT1 staining is in macrophages surrounding a necrotic area (*asterisk*). The lower middle and right images are higher magnifications of the area indicated by the box in the low-magnification CD68 image. These lower middle image shows the result of double immunostaining with anti-Ser-P-STAT1 (*dark, punctate structures*) and anti-CD68 (*red*), demonstrating Ser-P-STAT1 in the nuclei of macrophages. The lower right image shows the result of double immunostaining with TUNEL (*dark, punctate structures*) and anti-CD68 (*red*), demonstrating apoptotic macrophages. Nuclei of non-apoptotic cells are stained green. Note that exact alignment of the nuclei is not possible due to the fact that the sections are from separate tissue slices.

Figure 8. STAT1 plays a role in advanced lesional macrophage apoptosis and plaque necrosis in female *Stat1*-/- → *Ldlr*-/- mice. (***A***) The *table* shows plasma cholesterol and body weight of *Ldlr*-/- mice transplanted with *Stat1*+/+ or *Stat1*−/− bone marrow and then fed a Western-type

diet for 10 wks starting at 6 wks after transplantation. The *graph* shows pooled plasma samples from three *Stat1*+/+ and three *Stat1*-/- recipient mice that were fractionated by FPLC gel-filtration chromatography and then assayed for cholesterol. None of the differences in cholesterol, lipoproteins, or body weight were statistically significant. (***B***) Hematoxylin and eosin staining of proximal aortas from 10-wk Western diet-fed *Ldlr*-/- mice transplanted with bone marrow from *Stat1*+/+ and *Stat1*-/- mice. *Bar*, 20 μm. *Nec* = necrotic areas. (***C***) TUNEL (red), DAPI (blue), and macrophage (brown) staining of lesions similar to those in (B). *Bar*, 20 μm. The graph in panel C shows quantification of lesion area, TUNEL-positive cells, and necrotic area in the lesions of *Stat1*+/+ → *Ldlr*-/- and *Stat1*-/- → *Ldlr*-/- mice. *, p = 0.034 by Student's *t* test. (***D***) Hematoxylin and eosin staining of proximal aortas from 12-wk Western diet-fed *Ldlr*-/- mice transplanted with bone marrow from *Stat1*+/+ and *Stat1*-/- mice (n = 18 for both groups of mice). *Bar*, 20 μm. The graph in panel D shows quantification of TUNEL-positive cells and necrotic area in the lesions of *Stat1*+/+ → *Ldlr*-/- and *Stat1*-/- → *Ldlr*-/- mice. Total lesion area was 493.7 ± 40.5 and 380.6 ± 24.4 μm^2 in *Stat1*+/+ → *Ldlr*-/- and *Stat1*-/- → *Ldlr*-/- mice, respectively. *, p = 0.02 by Student's *t* test.

Figure 9. Integration of calcium, CaMKII, and STAT1 into the multi-hit pathway of macrophage apoptosis. According to this working hypothesis, ER stress-induced increase in cytosolic calcium triggers two pro-apoptotic hits: UPR/CHOP and a pathway involving CaMKII and Ser-P-STAT1. TLR4 activation also contributes to STAT1 serine phosphorylation. Ser-P-STAT1 is depicted as a separate pathway from CHOP, because studies with *Chop*-/- and *Stat1*-/- macrophages showed that CHOP is neither upstream nor downstream of Ser-P-STAT1 (data not shown). See Discussion for details and for a description of the areas of uncertainty in this model.

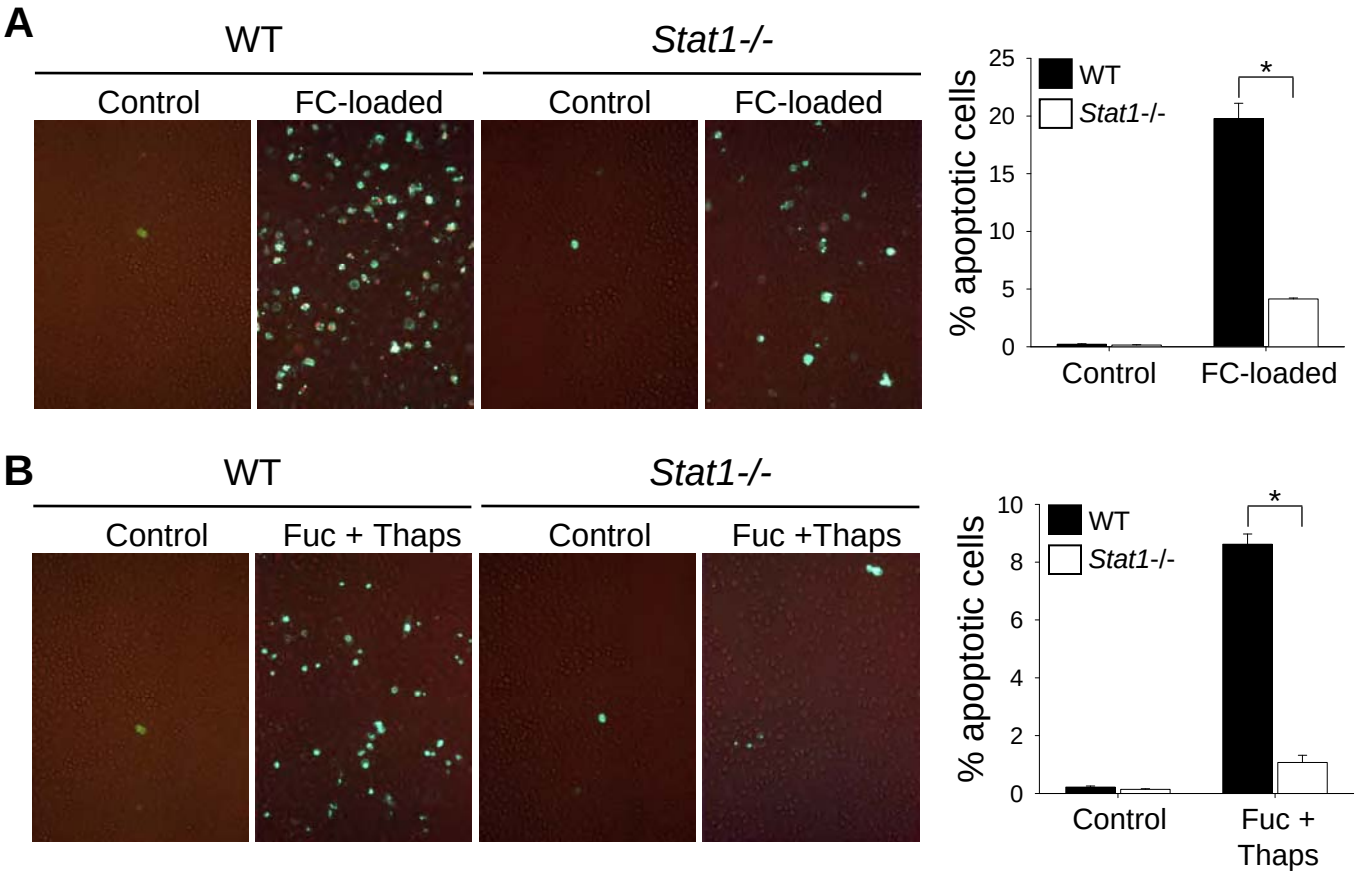

A
WT
Stat1-/-
Control
FC-loaded
Control
FC-loaded
% apoptotic cells
25
20
15
10
5
0
WT
Stat1-/-
*
Control
FC-loaded
B
WT
Stat1-/-
Control
Fuc + Thaps
Control
Fuc +Thaps
% apoptotic cells
10
8
6
4
2
0
WT
Stat1-/-
*
Control
Fuc + Thaps
C
WT
Stat1-/-
FC loading (h)
0 2 4 8 12 16
0 2 4 8 12 16
CHOP
Total Stat1
β-actin

Figure 1

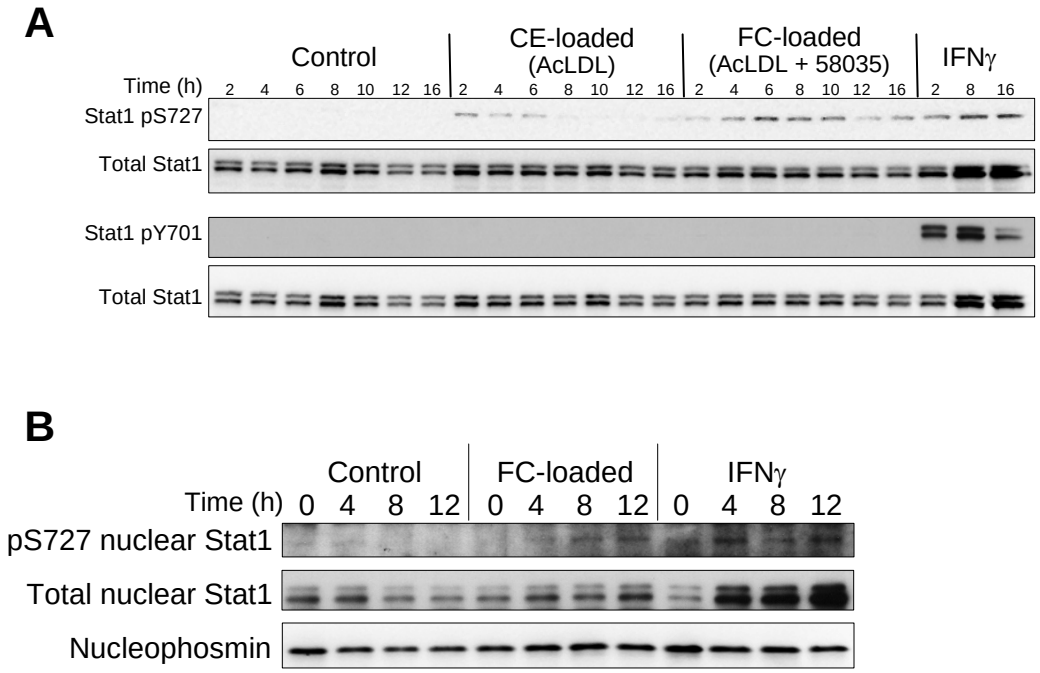

A
Control
CE-loaded
(AcLDL)
FC-loaded
(AcLDL + 58035)
IFNγ
Time (h)
2 4 6 8 10 12 16 2 4 6 8 10 12 16 2 4 6 8 10 12 16 2 8 16
Stat1 pS727
Total Stat1
Stat1 pY701
Total Stat1
B
Control
FC-loaded
IFNγ
Time (h) 0 4 8 12 0 4 8 12 0 4 8 12
pS727 nuclear Stat1
Total nuclear Stat1
Nucleophosmin

Figure 2

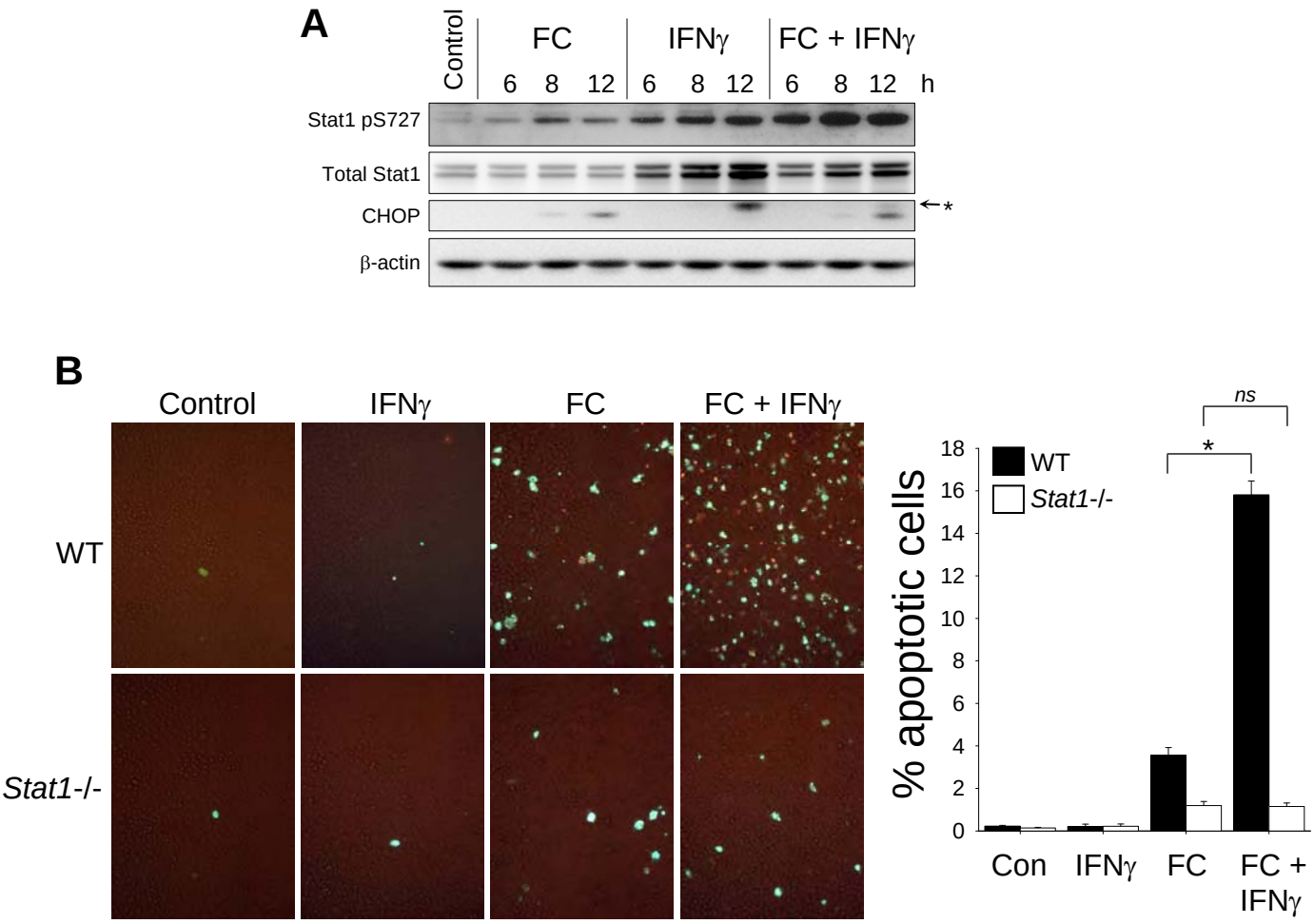

A
Control
FC
IFNγ
FC + IFNγ
6 8 12 6 8 12 6 8 12 h
Stat1 pS727
Total Stat1
CHOP
β-actin
*
B
Control
IFNγ
FC
FC + IFNγ
WT
Stat1-/-
% apoptotic cells
WT
Stat1-/-
ns
*
Con
IFNγ
FC
FC + IFNγ

Figure 3

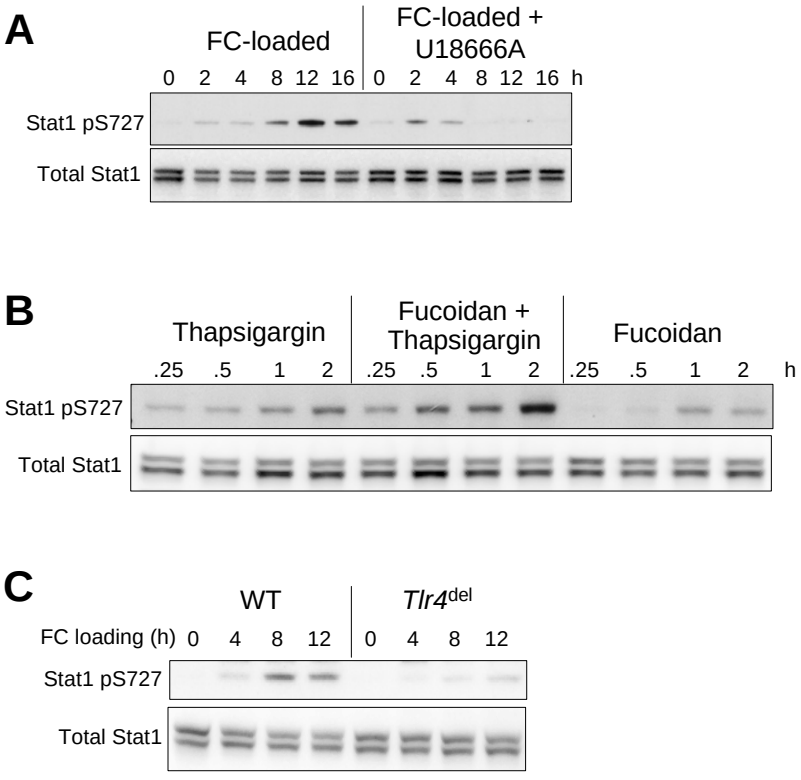

A
FC-loaded
FC-loaded + U18666A
0 2 4 8 12 16 0 2 4 8 12 16 h
Stat1 pS727
Total Stat1
B
Thapsigargin
Fucoidan + Thapsigargin
Fucoidan
.25 .5 1 2 .25 .5 1 2 .25 .5 1 2 h
Stat1 pS727
Total Stat1
C
WT
Tlr4del
FC loading (h) 0 4 8 12 0 4 8 12
Stat1 pS727
Total Stat1

Figure 4

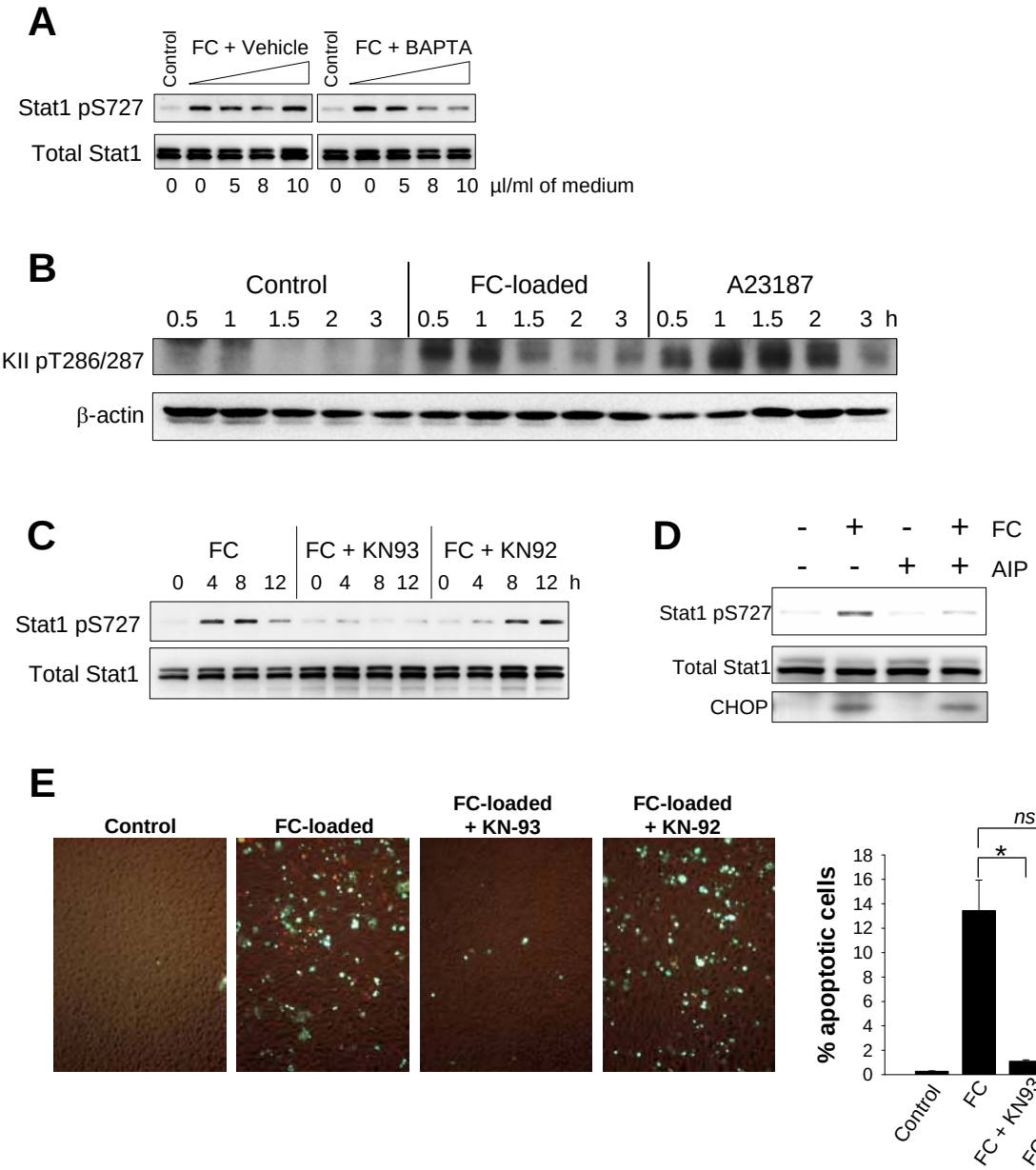
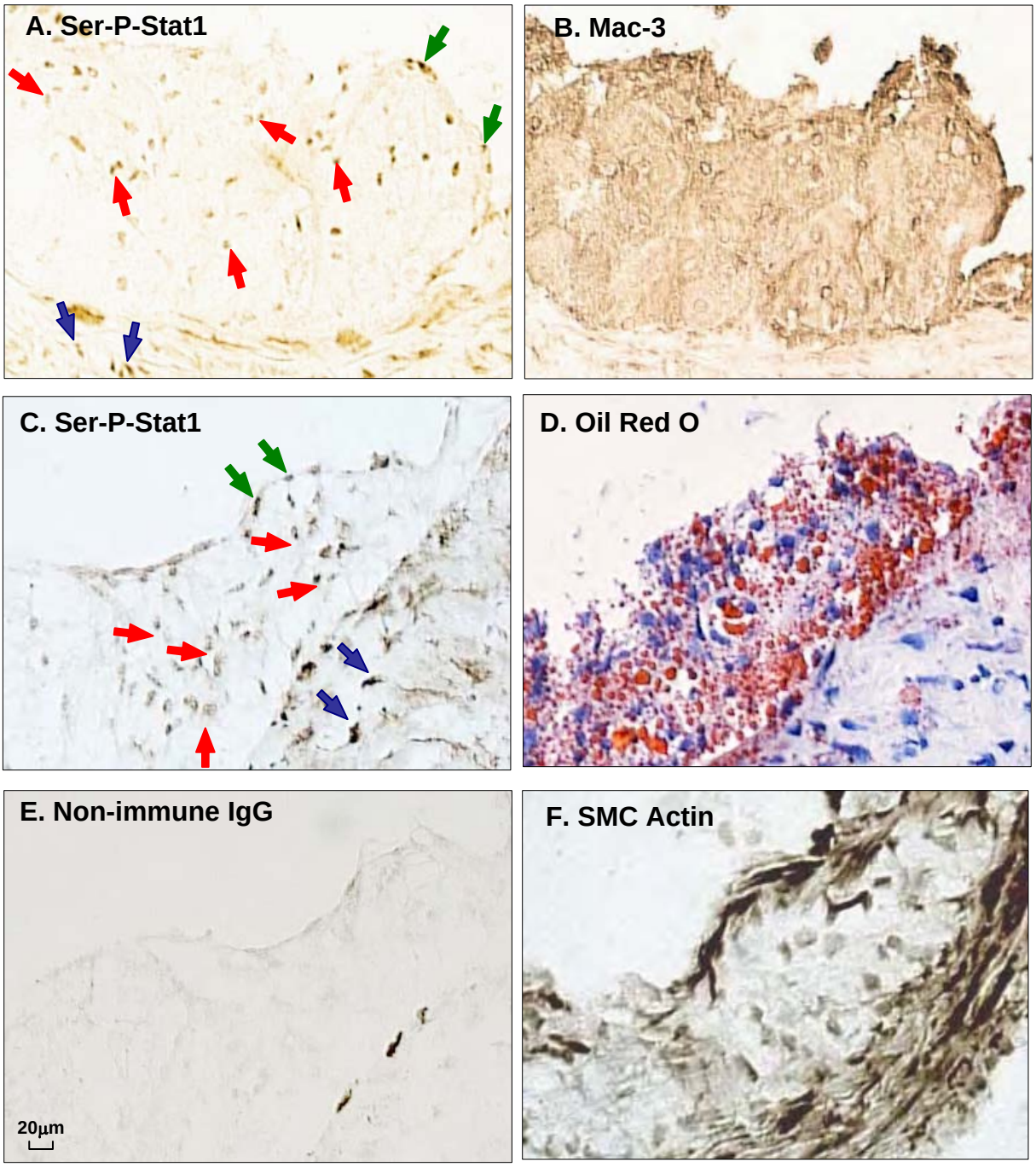

A
Control
FC + Vehicle
Control
FC + BAPTA
Stat1 pS727
Total Stat1
0 0 5 8 10 0 0 5 8 10 µl/ml of medium
B
Control
FC-loaded
A23187
0.5 1 1.5 2 3 0.5 1 1.5 2 3 0.5 1 1.5 2 3 h
CaMKII pT286/287
β-actin
C
FC
FC + KN93
FC + KN92
0 4 8 12 0 4 8 12 0 4 8 12 h
Stat1 pS727
Total Stat1
D
- + - + FC
- - + + AIP
Stat1 pS727
Total Stat1
CHOP
E
Control
FC-loaded
FC-loaded + KN-93
FC-loaded + KN-92
% apoptotic cells
ns
*
0 2 4 6 8 10 12 14 16 18
Control
FC
FC + KN93
FC + KN92

Figure 5

Figure 6


A. Ser-P-Stat1
B. Mac-3
C. Ser-P-Stat1
D. Oil Red O
E. Non-immune IgG
20μm
F. SMC Actin

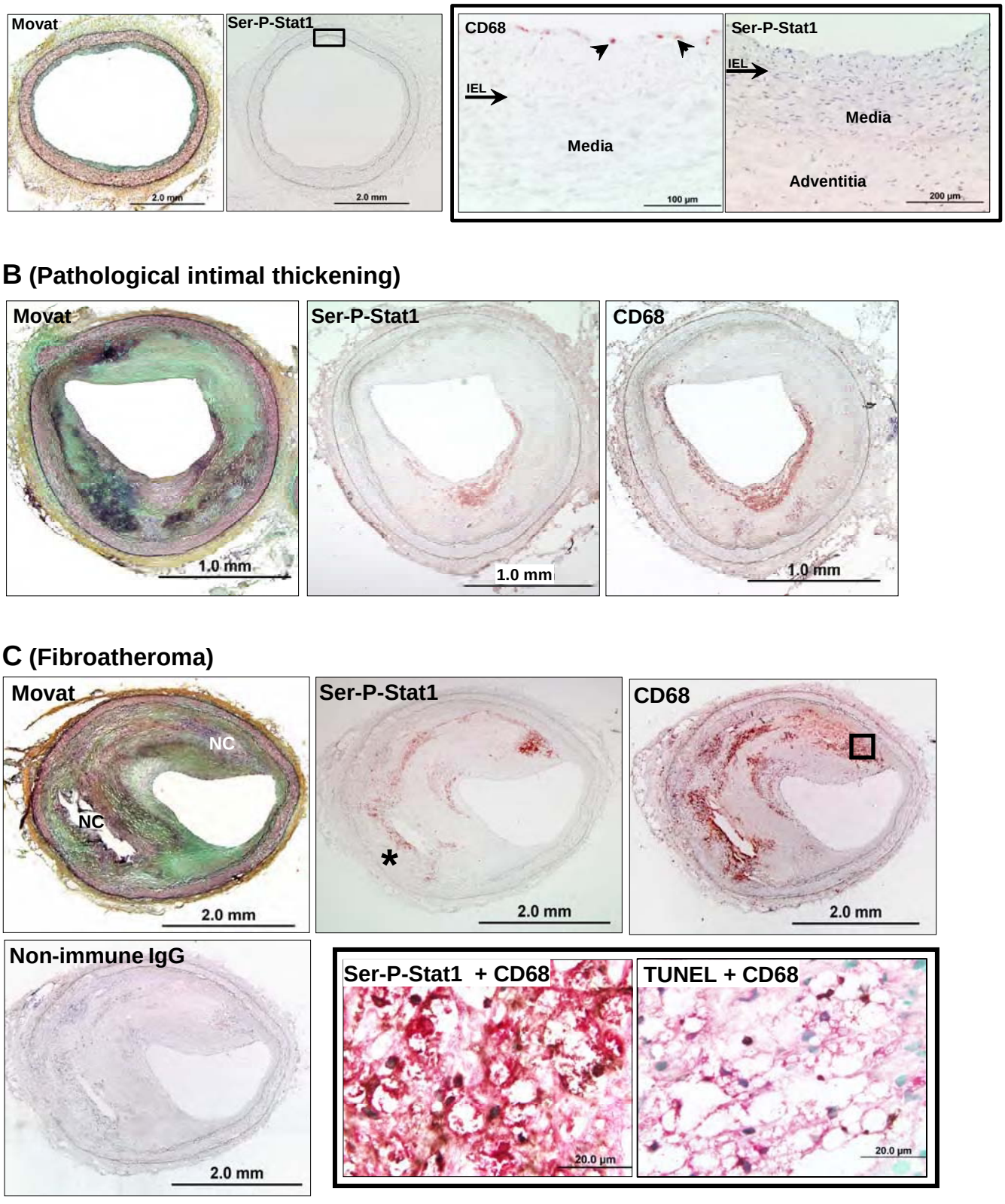
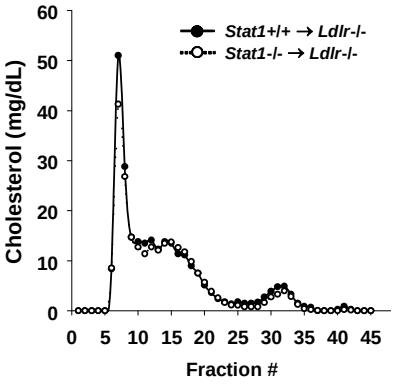

A (Diffuse intimal thickening)
Movat
2.0 mm
Ser-P-Stat1
2.0 mm
CD68
IEL
Media
100 µm
Ser-P-Stat1
IEL
Media
Adventitia
200 µm
B (Pathological intimal thickening)
Movat
1.0 mm
Ser-P-Stat1
1.0 mm
CD68
1.0 mm
C (Fibroatheroma)
Movat
NC
NC
2.0 mm
Ser-P-Stat1
*
2.0 mm
CD68
2.0 mm
Non-immune IgG
2.0 mm
Ser-P-Stat1 + CD68
20.0 µm
TUNEL + CD68
20.0 µm

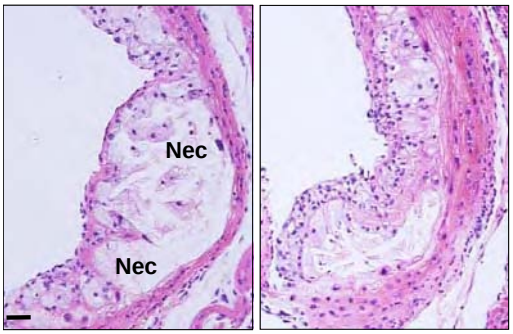
Figure 7

A

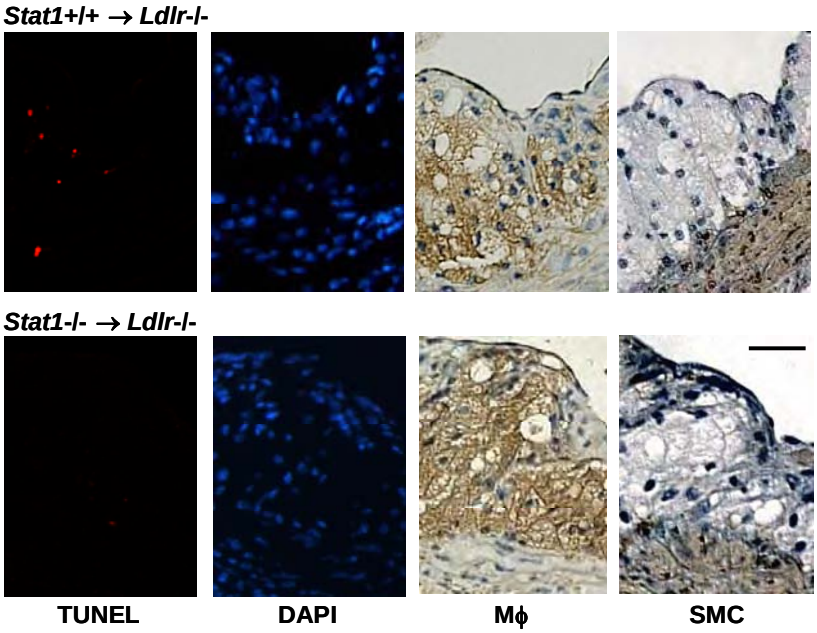
	Stat1+/+ → Ldlr-/- (n = 13)	*Stat1-/- → Ldlr-/-* (n = 9)
T. Chol (mg/dl)	636.5 ± 59.8	597.8 ± 63.8
HDL-Chol (mg/dl)	55.6 ± 3.2	56.1 ± 4.0
Body wt. (g)	20.0 ± 0.6	20.0 ± 0.8

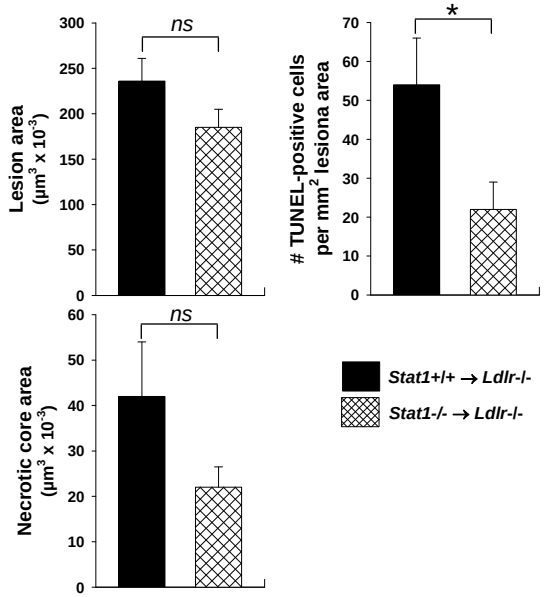


B

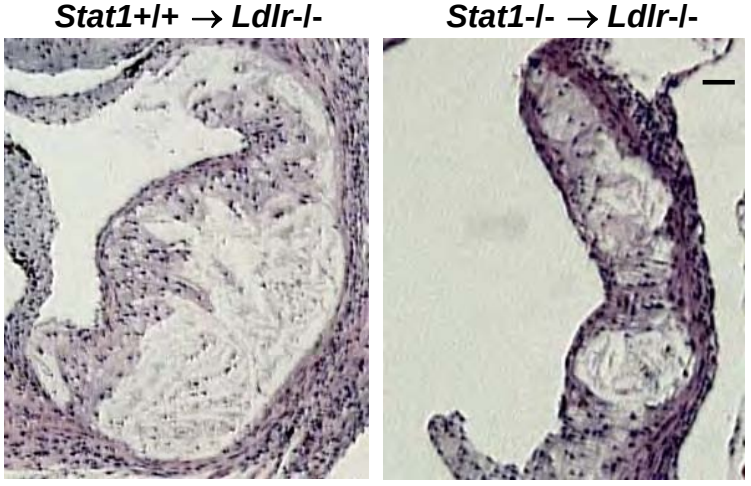


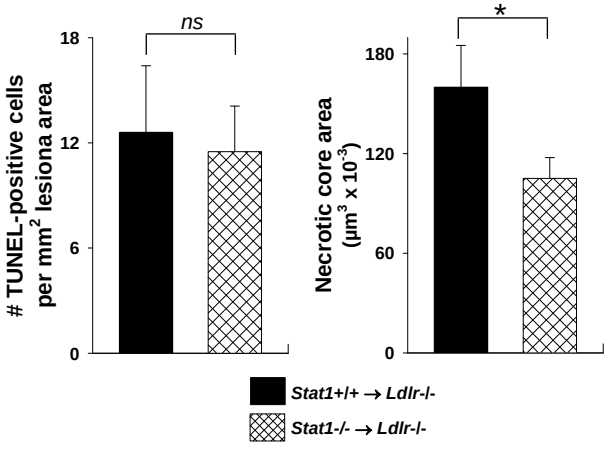
C





D (12 wk Western diet)





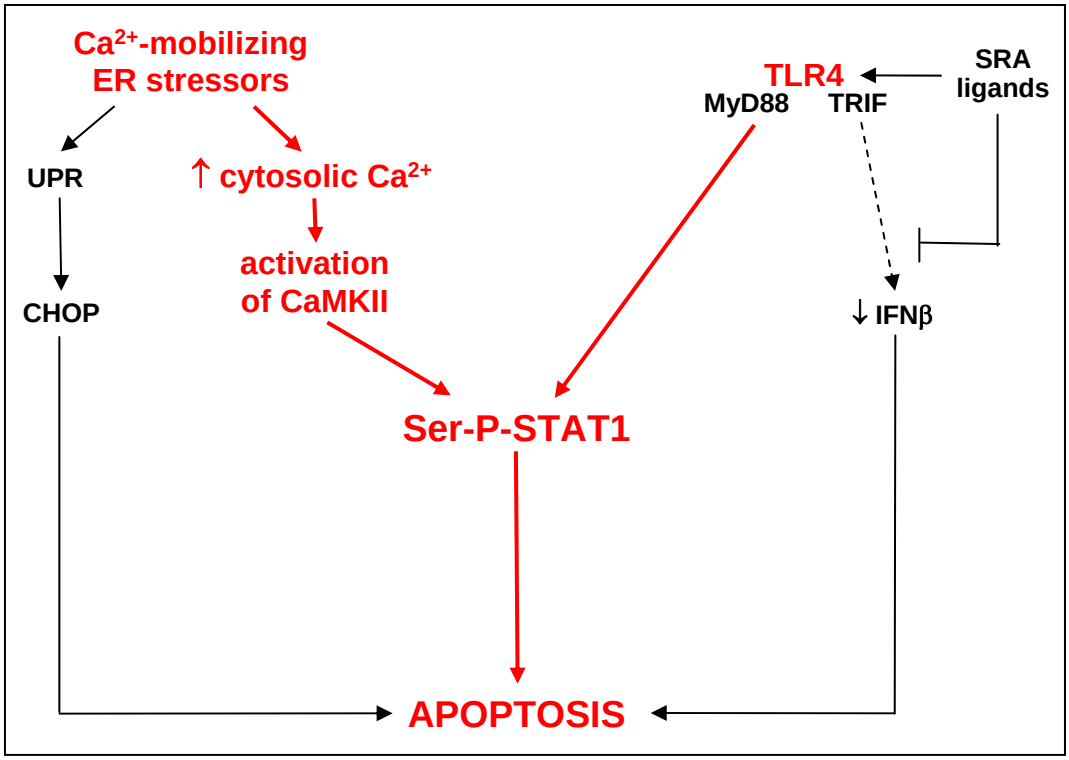

Ca2+-mobilizing
ER stressors
UPR
↑ cytosolic Ca2+
activation
of CaMKII
CHOP
TLR4
MyD88
TRIF
SRA
ligands
↓ IFNβ
Ser-P-STAT1
APOPTOSIS

Figure 9

SUPPLEMENTAL METHODS (Lim *et al*.)

Materials

Tissue culture media, cell culture reagents, and heat-inactivated FBS (GIBCO BRL) were purchased from Invitrogen. The acyl-coenzyme A-cholesterol acyltransferase (ACAT) inhibitor 58035 (3-[decyldimethylsilyl]-N-[2-(4-methylphenyl)-1-phenylethyl]propanamide (1) was from J. Heider, formally of Sandoz (East Hanover, NJ). A 10-mg/ml stock was made in DMSO and used at a concentration of 10 μg/ml in all experiments. Recombinant mouse IFNγ was from the PBL Biomedical Laboratories. The Vybrant Annexin V/Propidium Iodide Apoptosis Assay kit #2 was from Molecular Probes. LDL (d, 1.020-1.063 g/ml) from fresh human plasma was isolated by ultracentrifugation (2). Acetyl-LDL was prepared from LDL by reaction with acetic anhydride, as described previously (3), and used at a concentration of 50 μg/ml in all experiments. All other chemical reagents, including tunicamycin, thapsigargin, concanavalin A, and fucoidan, were purchased from Sigma-Aldrich.

Endotoxin Testing

Reagents used in this study were tested for endotoxin contamination by using the Limulus Amebocyte Lysate (LAL) kit (Cambrex, Walkersville, MD) and found to have <0.06 EU/ml endotoxin at working dilutions.

Eliciting and Culturing Mouse Peritoneal Macrophages

Macrophages were obtained from 8–10-wk-old female C57BL/6J mice (Jackson Laboratory); *Stat1*-/- mice on a C57BL/6J background (4); or *Tlr4*^{del} mice on a C57BL/10ScNJ background and wild-type C57BL/10ScSnJ background (Jackson Laboratory). The macrophages were harvested either three days after intraperitoneal (i.p.) injection of concanavalin A (5) or after immunization with methyl-BSA (6). For the latter method, 2 μg/ml methyl-BSA in 0.9% saline was emulsified in an equal volume of complete Freund's adjuvant (CFA) (Difco). Mice were immunized intradermally with 100 μl of the emulsion. After 14 days, the immunization protocol was repeated, except incomplete Freund's adjuvant was used instead of CFA. Seven days later, the mice were injected i.p. with 0.5 ml PBS containing 100 μg methyl-BSA and then the macrophages were harvested 4 days after that by peritoneal lavage. All macrophages were

cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 20% L-cell conditioned medium for 48-72 h, at which point they were typically at ~90% confluency.

Immunoblot Analysis

Cell lysates were prepared by homogenization in 1X sample buffer containing 2% SDS, 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 50 mM DTT, and 0.01% bromphenol blue, and boiled at 100°C for 5 min. Cytosolic and nuclear extracts were isolated using the Nuclear Extraction Kit (Panomics) according to the manufacturer's protocol. Cell extracts were electrophoresed on 4–20% gradient SDS-PAGE gels (Invitrogen) and transferred to 0.45-μm nitrocellulose membranes. The membranes were blocked in Tris-buffered saline, 0.1% Tween 20 (TBST), containing 5% (w/v) nonfat milk at room temperature for 1 h and then incubated with primary antibodies diluted in TBST containing 5% (w/v) nonfat milk or 5% BSA (w/v) at 4 °C overnight. This incubation was followed by incubation with HRP-conjugated secondary antibodies at room temperature for 1 h. Proteins were detected by SuperSignal West Pico-enhanced chemiluminescent solution (Pierce Chemical Co.). When required, membranes were stripped with Restore Western Blot Stripping Buffer (Pierce Chemical Co.) for 15 min at room temperature and reprobed for β-actin (loading control) or other proteins. Antibodies against total STAT1, GADD 153 (CHOP), and α-tubulin were purchased from Santa Cruz Biotechnology, Inc. Antibodies against phospho-Thr286/287 CaMKII, CaMKII, phospho-Ser727 STAT1, and phospho-Tyr701 STAT1 were purchased from Cell Signaling Technology. Anti-nucleophosmin antibody was purchased from Zymed, and anti-β-actin mouse monoclonal antibody was from Chemicon International. The HRP-conjugated donkey anti–mouse and donkey anti–rabbit IgG secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

Plasma Cholesterol Assays

Total plasma cholesterol was determined using an enzymatic kits from Wako Chemicals GmbH. Plasma high-density lipoprotein (HDL) cholesterol was determined after dextran sulfate-Mg^{2+} precipitation of apoB-containing lipoproteins. Lipoprotein-cholesterol profiles were determined by FPLC gel-filtration fractionation consisting of two Superose 6 columns connected in series (Amersham Pharmacia).

Immunohistochemistry of Murine and Human Atherosclerotic Lesions

Ldlr-/- mice on the C57BL/6 background were fed a "Western-type" diet (21% anhydrous milk fat and 0.15% cholesterol from Harlan-Teklad; TD88137) for 10 weeks. After anesthetization, the hearts of the mice were perfused with PBS. The hearts and proximal aortae were harvested, perfused *ex vivo* with PBS, embedded in OCT compound, snap frozen in an ethanol-dry ice bath, and stored at −70°C. Sections of proximal aortae (6-μm thick) were prepared at −20°C by using a Microm Microtome Cryostat HM 505 E and placed on poly-L-lysine-coated slides (Fisher Scientific). The sections were then fixed in ice-cold acetone for 10 min, air dried for 10 min, and stored at −70°C until use. All of the following procedures were conducted at room temperature. To detect phospho-S727 STAT1, the frozen sections were washed 3 times in PBS for 2 min, blocked for 1 h with 10% normal donkey serum (Jackson ImmunoResearch), and incubated for 1 h with 2.7 μg/ml rabbit anti-phospho-S727 STAT1 (Cell Signaling #9177) or, as a negative control, 2.7 μg/ml nonimmune rabbit IgG (Jackson ImmunoResearch). After washing 3 times in PBS, the sections were incubated for 30 min with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch). Finally, the sections were incubated with streptavidin-horseradish peroxidase and the DAB chromogen (both from BD Pharmingen), mounted in Permount, and viewed with an Olympus IX 70 inverted microscope using a ×40 objective. Parallel sections were stained with anti-Mac-3 (BD Pharmingen), anti-α-actin (Abcam), and anti-PECAM-1 (Santa Cruz Biotechnology) antibodies to label macrophages, smooth muscle cells, and endothelial cells, respectively. To stain macrophages in parafinized sections used for *in-situ* TUNEL analysis, sections were deparaffinized, rehydrated, blocked for 1 h with 10% normal donkey serum (Jackson ImmunoResearch), and incubated overnight with a rabbit anti-mouse macrophage antibody (AIA31240, Accurate Chemical & Scientific Corp.). The subsequent steps were identical to that of staining frozen sections described above. To visualize foam cells, frozen sections were fixed with buffered formalin and stained with Oil Red O for neutral lipid and Harris hematoxylin for nuclei.

For the human coronary artery study, the heart of a patient who had died suddenly of coronary causes was obtained as described previously (7). Coronary segments (3-4 mm in length) were frozen, cryosectioned, and stored at –80°C until use. For immunostaining, the sections were thawed, fixed in cold acetone (-20^{o}C), air-dried, and stained with primary antibodies against phosphorylated STAT1 (Millipore, Lake Placid, NY, Catalog # 07-714,

dilution 1:300) or the macrophage marker CD68 (Dako, Carpinteria, CA, Catalog #M0814, dilution 1:600) for 1 hour. Primary antibodies were labeled using an EnVision^{+} System, peroxidase kit (Catalog # K4009) for 30 min. Negative control staining for STAT1 was performed with non-immune IgG (Dako, catalog # X0903) at a similar protein concentration. Finally the signal was visualized by a 3-amino-9-ethylcarbozole substrate-chromogen system producing a rose red color; the sections were counterstained with Gill hematoxylin. For co-immunostaining with anti-Ser-P-STAT1 and anti-CD68, anti-Ser-P-STAT1 was first visualized using diaminobenzidine (DAB) tinted with nickel chloride (black color). The sections were then immunostained for CD68 and visualized using an alkaline phosphatase ABC kit (Vector, Burlingame, CA) with Vector red; sections were then counterstained with methyl green.

Apoptosis was identified by TUNEL (terminal deoxynucleotidyl transferase [TdT]-mediated dUTP nick-end labeling) using TdT from VasoTACS, Trevigen, Gaithersburg MD. Human tonsil served as a positive control. The reaction produce was visualized with DAB tinted with nickel chloride. The section was then stained with CD68 at 1:600 for 1 hour and developed with alkaline phosphatase red substrate kit (Vector, Burlingame, CA). The sections were then counterstained with methylgreen.

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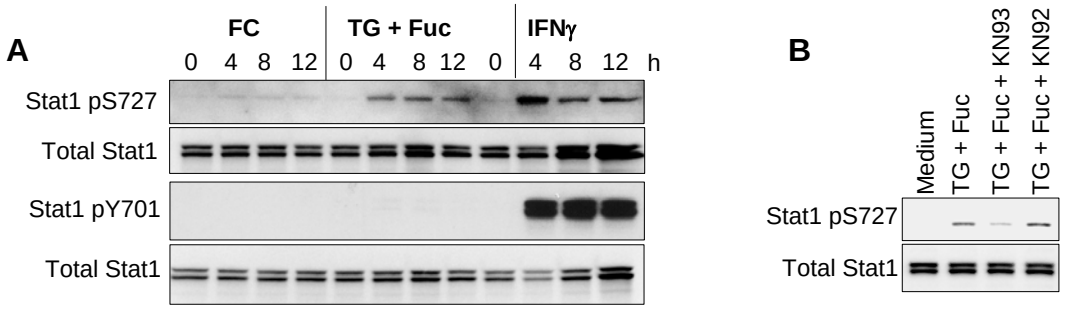
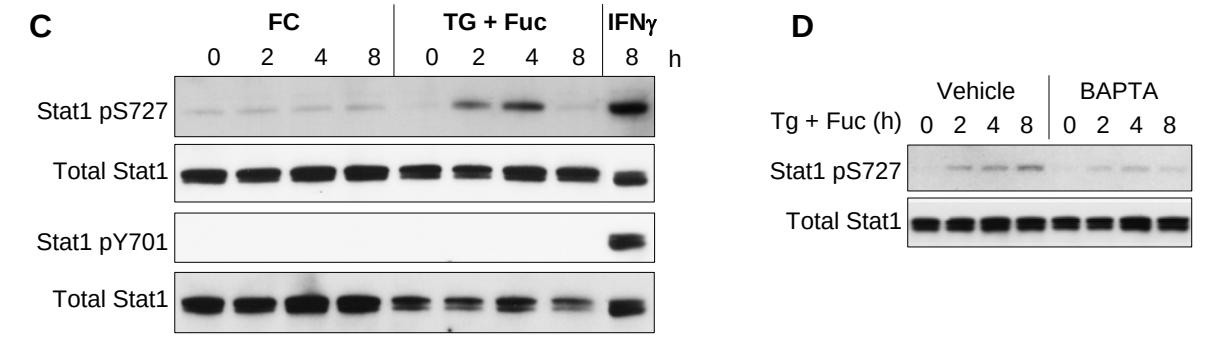
Mouse bone marrow-derived macrophages



Human monocyte-derived macrophages



Mouse bone marrow-derived macrophages (mBMDM) were collected as described in Methods and cultured for 7 days to permit differentiation into mature macrophages. **(*A*)** mBMDM were incubated with acetyl-LDL plus the ACAT inhibitor 58035 (*FC-loaded*), 50 μg/ml fucoidan and 0.5 μM thapsigargin (*TG+Fuc*), or 100 U/ml IFNγ for the indicated times. **(*B*)** mBMDM were incubated with 50 μg/ml fucoidan and 0.5 μM thapsigargin (*TG+Fuc*) plus 50μM KN93 or 50μM KN92 for 2 h.

Human monocyte-derived macrophages (hMDM) were isolated from normal human buffy coats as previously described (Bottalico *et al*. [1993] *J Biol Chem* 268: 8569-8573) and cultured in the presence of 1 ng/ml GM-CSF for 10 days. **(*C*)** hMDM were incubated with acetyl-LDL plus the ACAT inhibitor 58035 (*FC-loaded*), 50 μg/ml fucoidan and 0.5 μM thapsigargin (*TG+Fuc*), or 100 U/ml IFNγ for the indicated times. **(*D*)** hMDM were incubated with 50 μg/ml fucoidan and 0.5 μM thapsigargin (*TG+Fuc*) plus 10 μg/ml BAPTA or vehicle control for the indicated times. Whole-cell lysates were then subjected to immunoblot analysis to detect phospho-S727 STAT1 (*Stat1 pS727*), phospho-Y701 STAT1 (*Stat1 pY701*), and total STAT1.

CLINICAL PERSPECTIVE

Lim et al.

CIRCULATIONAHA/2007/711275

In industrialized societies, virtually all young adults have atherosclerosis. Most of these lesions are asymptomatic and will remain so for the rest of the person's life. However, a small percentage will progress to a dangerous stage involving plaque breakdown, acute lumenal thrombosis, and acute vascular events, like myocardial infarction and sudden cardiac death. Thus, a major goal is to elucidate the cellular-molecular events involved in benign-to-vulnerable plaque progression. A key feature of vulnerable plaques are "necrotic cores," which likely promote plaque breakdown and acute thrombosis. Necrotic cores are "graveyards of dead macrophages," a prominent cell type in atherosclerosis. Lim *et al*. used a cell-culture model of macrophage death to explore death-promoting molecules that may be relevant to advanced atherosclerosis. These experiments revealed an important role for a calcium-signaling pathway involving a two molecules called CaMKII and STAT-1. The authors showed that both mouse and humans advanced atheromata have activated STAT-1. Most importantly, when macrophages were made deficient in STAT-1 in a mouse model of advanced atherosclerosis, macrophage death and plaque necrosis were diminished. Two important caveats of this study need to be mentioned. First, the processes of macrophage death and plaque necrosis are complex, and so the CaMKII-STAT1 pathway represents only one piece of the puzzle. Second, the mouse is a poor model of plaque disruption and acute thrombosis. Thus, father studies will be needed to explore other pathways involved in advanced lesional macrophage death, and improved mouse models will be required to prove the hypothesis that macrophage death and plaque necrosis promote plaque disruption and acute thrombosis. Nonetheless, the Lim *et al*. study provides important new molecular-cellular information related to the progression of advanced atherosclerotic lesions—information that some day may be translated into therapy designed to block benign-to-vulnerable plaque transformation.

The impact of insulin resistance on macrophage death pathways in advanced atherosclerosis

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Abstract. Macrophage death in advanced atherosclerosis causes plaque necrosis, which promotes plaque rupture and acute atherothrombotic vascular events. Of interest, plaque necrosis and atherothrombotic disease are markedly increased in diabetes and metabolic syndrome. We discovered a novel 'multi-hit' macrophage apoptosis pathway that appears to be highly relevant to advanced atherosclerosis. The elements of the pathway include: (a) activation of the unfolded protein response (UPR) by cholesterol overloading of the endoplasmic reticulum or by other UPR activators known to exist in atheromata; and (b) pro-apoptotic signalling involving the type A scavenger receptor (SRA). The downstream apoptosis effectors include CHOP (GADD153) for the UPR and JNK for SRA signalling. Remarkably, components of this pathway are enhanced in macrophages with defective insulin signalling, including UPR activation and SRA expression. As a result, insulin-resistant macrophages show increased susceptibility to apoptosis when exposed to UPR activators and SRA ligands. Moreover, the advanced lesions of atherosclerosis-prone mice reconstituted with insulin-resistant macrophages show increased macrophage apoptosis and plaque necrosis. Based on these findings, we propose that one mechanism of increased plaque necrosis and atherothrombotic vascular disease in insulin resistant syndromes is up-regulation of a two-hit signal transduction pathway involved in advanced lesional macrophage death.

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Diet and lifestyle habits of industrialized societies have led to a situation where almost all adult individuals in these societies have variable numbers of subendothelial deposits of cholesterol, inflammatory cells and extracellular matrix in focal areas of the arterial tree (Braunwald 1997). The vast majority of these focal subendothelial deposits, called atherosclerotic lesions or plaques, are relatively small, stable and asymptomatic, and will remain so during the lifespan of the individual. However, in approximately 50% of this population, a small number of these

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atherosclerotic lesions will undergo a gradual transformation that can prove deadly. This transformation is characterized by changes in the morphology of the lesions that render them susceptible to erosion or rupture (Aikawa & Libby 2004, Kolodgie et al 2004, Libby 2000). If erosion or rupture does eventually occur in these susceptible, or 'vulnerable,' plaques, the exposure of thrombogenic plaque material to the bloodstream can lead to acute thrombotic vascular occlusion and infarction of the tissue supplied by the affected artery. In the heart, this scenario results in sudden cardiac death and/or myocardial infarction, and in the brain, this process can lead to certain types of stroke (Aikawa & Libby 2004, Kolodgie et al 2004, Libby 2000).

Understanding the mechanisms of stable-to-vulnerable plaque transformation at a cellular and molecular level will almost certainly suggest new therapeutic strategies to combat atherothrombotic vascular disease. Because both atherogenesis itself and the transformation to plaque vulnerability are complex processes, a reductionist approach is necessary to elucidate molecular/cellular mechanisms. In this context, we have focused on one of the more prominent cellular events associated with plaque vulnerability, namely, advanced lesional macrophage death. In early life, the arterial subendothelium is largely devoid of cells and extracellular lipids. However, by the early teen years, areas of the arterial tree characterized by disturbed blood flow begin to accumulate circulating lipoproteins in the subendothelial space (Williams & Tabas 1995, 1998). The most important of these lipoproteins are low density lipoproteins (LDL), derived from hepatic secretion of VLDL, and so-called remnant lipoproteins, derived from partial catabolism of intestinally derived chylomicrons and hepatic-derived VLDL (Williams & Tabas 1995, 1998). The key initiating event of subendothelial lipoprotein retention, which is influenced by the plasma level of these 'atherogenic' lipoproteins and poorly understood genetic factors at the level of the arterial wall, triggers a series of biological responses (Williams & Tabas 1995, 1998). The most prominent response to subendothelial lipoprotein retention is monocyte infiltration. The monocytes differentiate into macrophages in the subendothelial space, and the macrophages then ingest the retained lipoproteins by a variety of processes including endocytosis, pinocytosis and phagocytosis (Tabas 2002). Lipoprotein uptake by macrophages results in a large delivery of lipoprotein-derived lipids, particularly cholesterol. Although excess cholesterol can be toxic to cells, early lesional macrophages remain healthy by effluxing the cholesterol and by storing it in a relatively harmless form called cholesteryl fatty acid esters (Glass & Witztum 2001). This latter process, which gives rise to cytoplasmic lipid droplets and thus a 'foamy' appearance to the macrophages, is catalysed by an endoplasmic reticulum (ER) enzyme called acyl-CoA:cholesterol acyltransferase (ACAT) (Chang et al 2001). Early foam cell lesions tend be non-occlusive and stable against erosion or rupture,

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which is in part due to a 'protective' collagenous 'cap' that separates the lesion from the bloodstream (Aikawa & Libby 2004, Kolodgie et al 2004).

Results and discussion

The importance of macrophage death in advanced atherosclerotic lesions

As alluded to above, the vast majority of these foam cells lesions remain non-occlusive and stable throughout the life of the individual. Indeed, reversal of atherosclerotic risk factors, especially lowering of plasma LDL by drugs and dietary changes, can cause at least partial regression of these lesions (Williams & Tabas 2005). However, a few of the lesions, particularly in the setting of persistent risk factors such as high plasma LDL, cigarette smoking and diabetes, can progress to the aforementioned vulnerable plaque stage. One of the key features of vulnerable plaques are vast areas of macrophage debris, which result from death of lesional macrophages (Tabas 2005). These areas of dying macrophages and macrophage debris, often referred to as 'necrotic cores' or 'lipid cores,' almost certainly contribute to plaque erosion or rupture by promoting inflammation, physical stress on the fibrous cap, and thrombosis (Libby & Clinton 1993, Tabas 2005). It is likely that these processes promoted by dead macrophages complement those triggered by living macrophages, such as secretion of matrix proteases and inflammatory cytokines, to induce plaque breakdown and acute vascular thrombosis (Libby & Clinton 1993, Tabas 2005).

According to the above scenario, advanced lesional macrophage death may be a key cellular event in stable-to-vulnerable plaque transformation. However, simply relating macrophage death to necrotic core formation and worsening of plaques can be misleading (Fig. 1). Macrophages in all stages of atherosclerosis undergo a certain basal rate of turnover by the caspase-dependent death process known as apoptosis (Tabas 2005). In early lesions, these apoptotic macrophages are rarely seen, because they are rapidly and efficiently phagocytosed by neighbouring macrophages (Liu et al 2005, Tabas 2005). This process of apoptotic cell clearance via phagocytosis, often referred to as 'efferocytosis,' is a normal physiological response to apoptosis and is non-inflammatory and prevents cellular necrosis (Henson et al 2001). Indeed, promoting early lesional macrophage apoptosis by genetic manipulation in mouse models of atherosclerosis results in a decrease in lesion cellularity and size, because the lesional macrophages are safely decreased in number (Liu et al 2005). In advanced lesions, however, there is evidence that efferocytosis of apoptotic macrophages is less than completely efficient (Schrijvers et al 2005, Tabas 2005). Apoptotic cells that do not get rapidly cleared by efferocytosis become leaky and trigger an inflammatory response—a process referred to as post-

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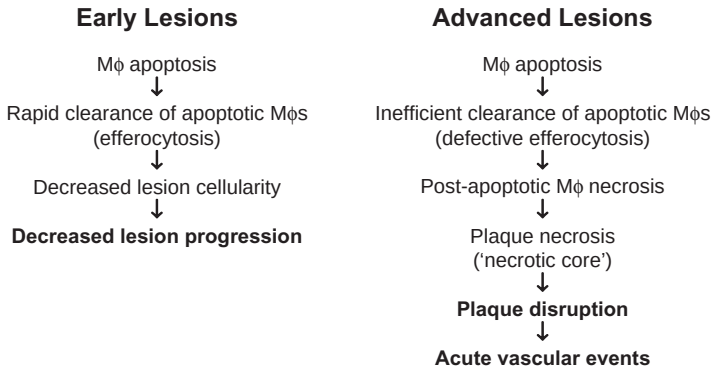

FIG. 1. Model of the opposing roles of macrophage death in atherosclerosis. In early lesions, apoptotic macrophages are rapidly cleared by efferocytosis, leading to reduced lesion cellularity and decreased lesion progression. In advanced lesions, however, efferocytosis is not as efficient, and so some of the apoptotic macrophages become secondarily necrotic. These necrotic macrophages gradually coalesce, forming the necrotic core. The combination of plaque necrosis, i.e. dead macrophages, and residual living macrophages (not shown) promotes plaque disruption and acute lumenal thrombosis. See text and Tabas (2005) for details.

apoptotic, or secondary, necrosis (Fiers et al 1999, Tabas 2005). By this manner, the necrotic core of advanced atherosclerotic lesions gradually forms.

The UPR-SRA model of advanced lesional macrophage apoptosis

Based on the above description of plaque progression, we feel that understanding the cellular/molecular basis of two key processes in advanced lesional macrophages—apoptosis and efferocytosis—is likely to shed new light into how stable atherosclerotic lesions transform into vulnerable plaques. For purposes of focus, this chapter will address the issue of how macrophages might undergo apoptosis in advanced atherosclerotic lesions. Our studies in this area began with a single model based on observations in human vulnerable plaques, but ensuing mechanistic studies of this model uncovered a much broader array of possible triggers that are likely to be relevant to atherosclerosis. The initial model was based on observations that the macrophages in vulnerable human plaques contain more unesterified, or 'free,' cholesterol (FC) than is typically observed in earlier lesional macrophage foam cells (see above) (Aikawa & Libby 2004, Burke et al 2003, Guyton & Klemp 1994, Kolodgie et al 2004, Kruth 1984, Lundberg 1985, Small 1988). Although the mechanism of FC accumulation is not known, it is likely promoted by dysfunctions of ACAT-mediated cholesterol esterification and cellular cholesterol efflux (above).

Based on this observation and the known cytotoxic effects of excess intracellular FC (Warner et al 1995, Yao & Tabas 2000, Yao & Tabas 2001), we sought to understand how FC accumulation would effect macrophages. The model we chose was one in which primary tissue macrophages (murine peritoneal macrophages) were exposed in culture to atherogenic lipoproteins in the setting of pharmacological or genetic ACAT dysfunction. We found that FC accumulation was a trigger for caspase-dependent macrophage apoptosis by pathways involving both the Fas death receptor and well-described mitochondrial apoptotic mechanisms (Yao & Tabas 2000, 2001). Initially, we imagined that excess FC in the plasma membrane and/or mitochondria might somehow trigger these events. However, our studies revealed that the key organelle was neither of the above but rather the ER (Feng et al 2003b, 2003a). In retrospect, the ER would have been a logical candidate, because the ER membrane bilayer normally has a relatively low cholesterol:phospholipid ratio. This property is responsible for the fluid nature of the ER membrane, which is necessary for its proper function (Davis & Poznansky 1987). When the cholesterol:phospholipid ratio of the ER membrane is increased, such as occurs during FC enrichment of macrophages, the membrane undergoes a phase transition to a more ordered state (Li et al 2004). This abnormal state leads to dysfunction of critical ER membrane proteins, including a protein called SERCA, which controls calcium levels in the ER (Li et al 2004).

The macrophage responds to this state of ER 'stress' by activating a coordinated signal transduction pathway known as the unfolded protein response (UPR) (Feng et al 2003a). The UPR is triggered by a wide variety of ER stressors, and its major function is to reverse the stress and to keep the ER protected while carrying out this repair function (Ma & Hendershot 2001, Ron 2002, Welihinda et al 1999). Thus, for example, protein translation is suppressed, unfolded proteins are degraded, and protein chaperones are induced (Ma & Hendershot 2001, Ron 2002, Welihinda et al 1999). However, if ER stress is prolonged or unable to be repaired, there is a distal branch of the UPR can promote apoptosis (McCullough et al 2001, Oyadomari et al 2002, Zinszner et al 1998). In many cases, including the situation with FC-enriched macrophages, the apoptotic function of the UPR is effected by the transcription factor called CHOP, or GADD153 (McCullough et al 2001, Oyadomari et al 2002, Zinszner et al 1998). Although the exact mechanism of CHOP-induced apoptosis is still be explored in our and other laboratories, CHOP-deficient macrophages are substantially protected against FC-induced apoptosis (Feng et al 2003a). Moreover, recent unpublished data in our laboratory suggest that advanced lesions of *Chop*$^{-/-}$ mice on an atherogenic background have less macrophage death and less plaque necrosis than *Chop*$^{+/+}$ mice on the same background.

The concept that the UPR is an apoptosis trigger is simplistic. In fact, the UPR normally promotes cell survival to enable repair (above), and apoptosis is induced

only when something else goes awry (Ma & Hendershot 2001, Ron 2002, Welihinda et al 1999). Thus, the UPR can, under certain circumstances, be necessary for apoptosis, but it is never sufficient by itself. Rather, the UPR can enable apoptosis in response to another 'hit.' This two-hit model of UPR-induced apoptosis is critical to understanding how FC enrichment kills macrophages. In this scenario, the UPR–CHOP pathway is necessary but not sufficient for FC-induced apoptosis. We found that the 'second hit' in this model involved engagement of a receptor for atherogenic lipoproteins called the type A scavenger receptor (SRA) (DeVries-Seimon et al 2005) (Fig. 2). This second hit was effected by the method we had chosen to deliver cholesterol to the macrophages, namely, because we had used a typical SRA-binding atherogenic lipoprotein to load the cells with cholesterol (DeVries-Seimon et al 2005). Thus, atherogenic lipoproteins induce death by satisfying both hits of the two-hit model: they deliver cholesterol to the ER to activate the UPR–CHOP pathway, and they engage the SRA to supply the second hit. We

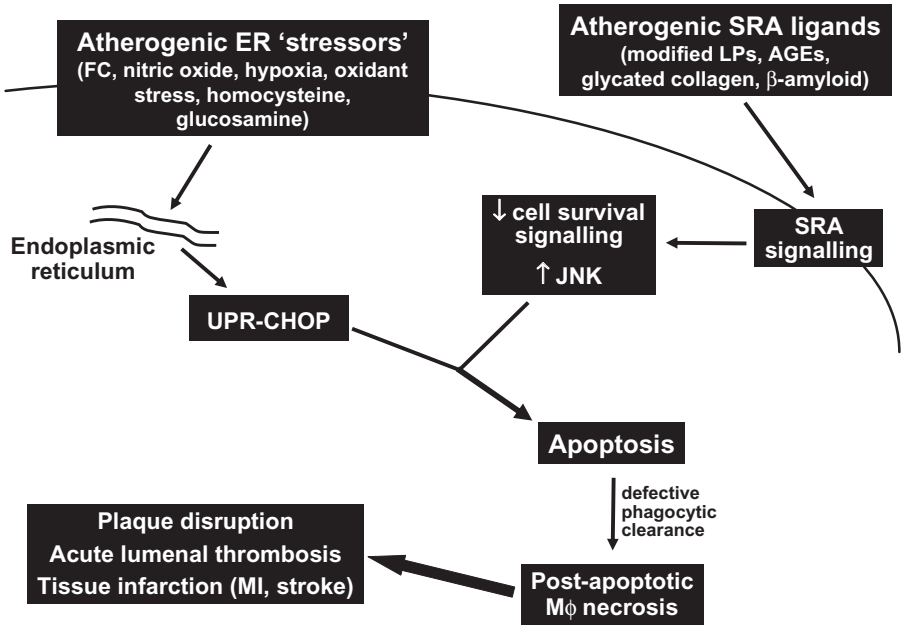

FIG. 2. The UPR–SRA two-hit model of macrophage apoptosis. UPR activation by FC loading or other UPR activators known to be present in advanced atherosclerotic lesions provides the *potential* for apoptosis through the UPR effector CHOP. However, apoptosis does not occur unless a second hit is present, which in the case of macrophage apoptosis is engagement of the SRA. Ongoing mechanistic suggest that the SRA both triggers a pro-apoptotic JNK pathway and inhibits a cell survival pathway.

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now know that any combination of UPR activators and SRA ligands, many of which exist in advanced atherosclerotic lesions (Fig. 2), can trigger macrophage apoptosis completely independently of FC enrichment and ACAT dysfunction. These findings are very important, because they extend the array of possible triggers of advanced lesional macrophage death beyond the FC enrichment/ACAT dysfunction model. How SRA engagement supplies the second apoptotic hit to ER-stressed macrophages is a key area of current focus in the laboratory. Our current data suggest a fascinating mechanism in which SRA engagement triggers activation of the pro-apoptotic mitogen-activated protein kinase JNK while selectively silencing a cell survival pathway in the macrophages.

The link to insulin resistance

Humans with type 2 diabetes have a markedly increased risk of atherothrombotic vascular disease (Plutzky et al 2002). Of significant relevance to the topic of this chapter, diabetic lesions are characterized by increased necrotic cores (Burke et al 2004), suggesting an enhancement of advanced lesional macrophage death. The increase in plaque progression in diabetics is likely due to a combination of insulin resistance and hyperglycaemia. Certainly, insulin resistance by itself is a strong risk factor, because humans with insulin resistance without hyperglycaemia, e.g. in metabolic syndrome, have an increased incidence of coronary artery disease (Grundy 2004).

There are a number of possible mechanisms whereby insulin resistance might promote plaque progression, including enhancement of systemic atherosclerotic risk factors, like low high-density lipoprotein (HDL) and hypertension, and direct effects on cells of the arterial wall, notably endothelial cells (Plutzky et al 2002). In a recent collaboration with Drs Alan Tall and Domenico Accili of Columbia University, we have been investigating another possible mechanism, namely, pro-apoptotic effects macrophage insulin resistance in the context of the UPR–SRA model described above. Although macrophages do not have insulin-regulatable glucose transporters, they do have insulin receptors that respond to insulin through the canonical insulin signalling pathway (Liang et al 2004). Thus, acute treatment of macrophages with physiologic insulin concentrations leads to phosphorylation of the insulin receptor, insulin receptor substrate 2 (IRS2), and Akt (Liang et al 2004). Most relevant to the topic of this discussion, macrophages isolated from insulin-resistant mouse models, such as the hyperinsulinaemic leptin-deficient *ob/ob* mouse, have down-regulated insulin receptors and depressed insulin signaling (Liang et al 2004). Thus, macrophages, like hepatocytes and skeletal muscle cells, become insulin resistant in the setting of hyperinsulinaemia *in vivo*.

Remarkably, two of the more prominent characteristics of macrophages in the insulin-resistant state are activation of the UPR and up-regulation of the SRA (Han

et al 2006, Liang et al 2004). Although the molecular mechanisms behind these two processes are still under investigation, SRA up-regulation appears to be post-transcriptional and causally related to UPR activation (Han et al 2006, Liang et al 2004). These findings directly suggested that insulin-resistant macrophages would be more susceptible to the model of apoptosis described above. Indeed, we found that such macrophages show markedly enhanced apoptosis when exposed to SRA-mediated FC enrichment conditions or when treated with the combination of a UPR activator and an SRA ligand (Han et al 2006). Most importantly, we have data supporting this model in advanced atherosclerotic lesions. Specifically, we found that the advanced lesions of *Ldlr*$^{-/-}$ mice have increased macrophage apoptosis and lesional necrosis when they are reconstituted with bone marrow from insulin-resistant mice (Han et al 2006).

Studies are ongoing to determine whether the increased apoptosis observed in insulin-resistant macrophages represents an enhancement of the same UPR-SRA pro-apoptotic signalling pathway used by insulin-sensitive macrophages (above), as suggested by the UPR activation and SRA up-regulation in these cells (Fig. 3), or whether the insulin-resistant state triggers a second pro-apoptotic pathway that

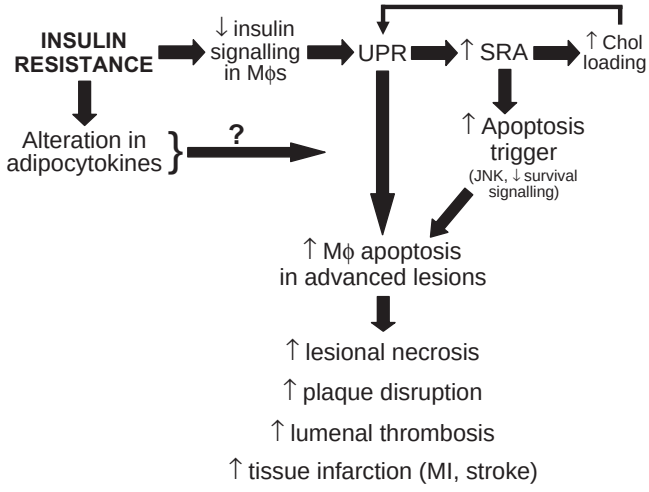

FIG. 3. Model of how insulin resistance promotes advanced lesional macrophage apoptosis. Macrophage insulin resistance directly activates the UPR, which in turn leads to up-regulation of the SRA. Each of these processes contributes to apoptosis by the two-hit model outlined in Fig. 2. In addition, the up-regulation of the SRA might promote enhanced internalization of lipoprotein-derived cholesterol, which might further activate the UPR in the setting of dysfunctional ACAT and suppressed cholesterol efflux. We are also exploring the possibility that alterations in adipocytokines in the setting of systemic insulin resistance might affect advanced lesional macrophage apoptosis.

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is additive or synergistic with the 'basal' pathway. Moreover, the laboratory is investigating molecular links between *systemic* insulin resistance and advanced lesional macrophage apoptosis, such as those that may be mediated by circulating adipocytokines that are altered in insulin-resistant states.

Summary and conclusions

Advanced lesional macrophage death is a key event in the transformation of asymptomatic atherosclerotic lesions into plaques that have the potential to rupture and cause acute vascular events. While multiple mechanisms are likely responsible for advanced lesional macrophage death, *in vivo* evidence suggests that a two-hit pro-apoptotic pathway involving the UPR and the SRA plays an important role. Apoptosis in this two-hit model results from pro-apoptotic CHOP from the UPR branch and pro-apoptotic JNK, plus suppression of cell-survival signalling, from the SRA branch. Remarkably, both of these hits are up-regulated in macrophages that are insulin resistant, and an atherosclerotic mouse models with insulin-resistant macrophages shows evidence of increased advanced lesional macrophage death and plaque necrosis. Further studies are needed to define exactly how defective insulin signalling in macrophages leads to UPR activation and SRA up-regulation; whether these events are responsible for the advanced lesional macrophage death observed *in vivo*; and whether other events associated with insulin resistance, such as alterations in adipocytokines, might contribute to this process.

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DISCUSSION

O'Rahilly: I was interested in the data generated from insulin receptor null cells. I would advise caution about using them as the unique model. If I had used this as my sole model for insulin resistance I would have got the story of insulin and adiponectin completely wrong. If you are thinking of the macrophages in an insulin resistant milieu, it is hard to know which cells are being exposed to a hyperinsulinaemic environment because they are not primarily insulin resistant themselves. The absolute absence of insulin may not be what they are seeing *in vivo*.

Tabas: The point you are raising is exactly why we designed the experiment that way. It was a reductionist approach to try to get at the bare mechanism of what is going on. You are right, but everything I have shown *in vitro* has been completely reproduced with Ob/Ob. We are now going back *in vivo* and doing everything with Ob/Ob. However, the reason we designed the first experiment the way we did is because of the points you raised. This model is unique. It is amazing how many

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people don't get this: insulin receptor down-regulation is an inherent part of insulin resistance. Our goal wasn't to try to attack the whole thing at first, but to look at what would happen if by itself we decrease insulin signalling in macrophages. Our second goal is to put this in a larger picture, as you are suggesting.

Spiegelman: I know that Ob/Ob mice have down-regulated insulin receptors, but I was under the impression that this wasn't generally true in humans with obesity and insulin resistance.

O'Rahilly: They do have down-regulated insulin receptors in a variety of cells. We haven't quantified the pattern precisely. In the average patient with insulin resistance, there are certainly some cells in the body which are seeing too much insulin action. The dermal fibroblast response of acanthosis nigricans which is commonly seen is almost certainly a hypertrophic response of cells to insulin, either occurring through its own receptor, hybrids or the insulin-like growth factor (IGF) receptor. Working out which tissues are seeing too much and which are seeing not enough insulin in any individual circumstance of hyperinsulinaemia is hard.

Tabas: We have data from human liver. These show a straight-line correlation between the level of insulin resistance and insulin receptors on hepatocytes. In the most insulin resistant states the insulin receptors in the hepatocytes are undetectable by Western blot.

Hotamisligil: It has been known for a long time that the down-regulation of insulin receptor quantity is insufficient to explain the extent of insulin resistance, which was the original observation that led to the examination of insulin receptor signalling. This is a critical phenomenon that is often overlooked. There is not insulin resistance uniformly in the body: some cells are seeing too much, some parts of insulin signalling are more active than others.

Tabas: This is something we have known for a long time. Brown & Goldstein (Shimomura et al 2000) brought this up in the liver where their feeling is that the insulin pathway that is involved in fatty acid metabolism and triglyceride synthesis remains hyperactive because of the high levels of circulating insulin. One of the reasons why the knockout is advantageous to the point we are bringing out is because it has zero insulin receptors. But I want to emphasize that the knockout was done at stage 1 in our study: we wanted to take a reductionist approach. Stage 2 is to put this in a larger context.

Hotamisligil: In the macrophage-specific insulin receptor knockout model versus the total knockout bone marrow transplantation model, are there differences between those experiments? There was also an IRS2 knockout, and looking at the total lesion area, it looked completely the other way (Han et al 2006, Baumgartl et al 2006). This is a little confusing.

Tabas: You shouldn't be confused at all. If you go back and look at the data in that paper and the data in our paper, the differences are really not the big. We

show a little but of an increase; in that paper there was somewhat of a decrease. In my mind that is not the story. The story, if there is a story at all, will be in the advanced lesional morphology events. The slight increase in lesion area we got and the moderate decrease in the IRS2 knockout is noise and it is not the most important part of the story.

Hotamisligil: I am intrigued by your comment that lesion size is less of a story.

Tabas: This is my bias. In humans, even though we now use coronary angiography as our gold standard, there is not a great correlation between lesion size and events. The best thing that angiography is doing is showing us these lesions that are associated with the dangerous lesions, which are known to be much more moderate in size than the larger lesions. The lesson we have learned from humans is that lesion morphology is more important than lesion size. It is on the basis of that knowledge that I have created a bias in using the mouse as a model that we are much more interested in advanced lesional morphology.

Spiegelman: The data you showed where adiponectin was suppressing UPR looks extremely impressive. To what extent can you hypothesize that this is the molecular basis of adiponectin action in metabolism? And have you examined which of the known adiponectin receptors is involved in that response?

Tabas: Could this be the answer to all the actions of adiponectin? We don't know. It is robust, and it was revealing for me to get Philipp Scherer's perspective. I am interested in atherosclerosis and macrophages, and he says, 'Ira, you have an *in vitro* assay that can distinguish between the high and low molecular weight forms!' It is so discriminatory between the potent and non-potent forms. We have been focused on macrophages so far and have tried to do just a few pilot experiments with some liver cell lines.

Spiegelman: You don't want to use liver cell lines, but primary hepatocytes.

Tabas: Could this be important in some of the other effects of adiponectin, particularly in view of what Gökhan Hotamisligil has taught us about how suppression of the UPR can improve insulin resistance? We know that high molecular weight adiponectin has been associated with improvement in insulin resistance, so could it all fit together? One of the reasons I was so excited about being at this meeting is so we could talk about this.

Hotamisligil: This is extremely exciting. It could not only explain why adiponectin is increasing insulin sensitivity, but it could also represent a nice loop, with increased UPR because of adiponectin and ER stress itself might be related to why there is less adiponectin. Adiponectin is one of the most complex molecules assembled in the ER. I would bet that if you induce ER stress, this blocks adiponectin synthesis.

Tabas: That's a good point. If adiponectin is made mostly by adipocytes, why do obese people have less adiponectin? Philipp Scherer has two ideas, one of which is exactly what you said. His other idea is related to inflammatory cytokines. D2

Spiegelman: That's a gene expression change.

Tabas: He thinks that two things are going on. He thinks the transcriptional is related to cytokines, and the post-transcriptional may be receptors. Remember, my collaborator is Philipp Scherer. This is the world I live in and the reagents I have available. His single knockouts don't show much, but the R1 and R2 double knockout is lethal. Professor Kadowaki finds small effects in R1 and R2 knockouts but his double knockout is viable, probably because there is a little leak through. He finds changes in insulin signalling. We have taken the R1 knockout and subjected it to R2 RNAi, getting 80% suppression. There is no diminution of the effect I have shown. We have no evidence at this point that R1 and R2 are involved. I am not sure what adiponectin is doing. We also know that AMPK is not involved. I flew my postdoc to Benoit Viollet in Paris. It turns out that macrophages only express the α1 form of adiponectin. There is no effect at all knocking out α1. As an outsider to this field I'm making no assumptions about the role of adiponectin. It may not even be the molecule itself that is doing something: it has lipid pockets so it could be delivering something.

Hotamisligil: Perhaps it is acting as a chaperone, providing it gets into the cell.

Tabas: The reason it isn't acting like a chaperone is that there is no chaperone that would not suppress eIF2α phosphorylation.

Hotamisligil: What if it is inhibiting an eIF2α phosphatase and acting as a chaperone?

Tabas: That's possible but unlikely in my opinion.

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DRUGS AFFECTING LIPID METABOLISM ANNUAL CONFERENCE
October, 2007, New York City

The Impact of Obesity and Insulin Resistance on Macrophage Apoptosis in Advanced Atherosclerosis

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Columbia University, New York, NY

Macrophage death in advanced atherosclerosis causes plaque necrosis, which promotes plaque disruption and acute atherothrombotic vascular events. Of interest, plaque necrosis and atherothrombotic disease are markedly increased in diabetes and metabolic syndrome. We discovered a "multi-hit" macrophage apoptosis pathway that appears to be highly relevant to advanced atherosclerosis. The hits include **(1)** a pro-apoptotic branch of the endoplasmic reticulum stress pathway known as the Unfolded Protein Response (UPR), which is mediated by the UPR effector CHOP; **(2)** a pathway involving the MAP kinase JNK, which is activated by combinatorial pattern recognition receptor signaling involving the type A scavenger receptor (SRA) and toll-like receptor 4; and **(3)** a pathway involving cytosolic calcium, calcium/calmodulin-dependent protein kinase II (CaMKII), and STAT1. Macrophages with defective insulin signaling show enhanced components of this pathway and increased susceptibility to apoptosis when exposed to UPR activators and SRA/TLR4 ligands. Moreover, the advanced lesions of atherosclerosis-prone mice reconstituted with insulin-resistant macrophages show increased macrophage apoptosis and plaque necrosis. Finally, a number of adipocytokines that are altered in obesity have profound effects on this multi-hit pathway of macrophage apoptosis. Based on these findings, we propose that one mechanism of increased plaque necrosis and atherothrombotic vascular disease in insulin resistant syndromes is amplification of a multi-hit signal transduction pathway involved in advanced lesional macrophage death.

Support: NIH grants HL75662 and HL54591 and USA Medical Research and Material Command Grant PR054352.

CURRICULUM VITAE

I. Date of preparation

December, 2007

II. Personal data

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III. Academic training

Undergraduate:	Tufts University (Medford, MA), 1975, B.S.
Graduate:	Washington University (St. Louis, MO), 1981, M.D., Ph.D. (Biochemistry)
Ph.D. Thesis:	"The Processing of Asparagine-Linked Oligosaccharides During Glycoprotein Biosynthesis"; Dr. Stuart Kornfeld, Sponsor
M.D. Licensure:	State of New York (#150522)

IV. Traineeship

Internship/Residency:	Internal Medicine, Columbia-Presbyterian Medical Center, New York, NY (1981-1983)
Clinical Fellowship:	Endocrinology/Metabolism, Columbia-Presbyterian Medical Center, New York, NY (1983-1985)
Research Fellowship:	Laboratory of Dr. Alan Tall, Department of Medicine, Columbia University, New York, NY, 1983-1985

V. Board certification

Internal Medicine, 1985
Endocrinology/Metabolism, 1987

VI. **Professional organizations and societies**

Arteriosclerosis Council (Arteriosclerosis, Thrombosis, and Vascular Biology as Council as of 1997) of the American Heart Association; appointed member of the Membership/ Credentials Committee (1990-1992, 1997-1999) and Program Committee (1992-1994; 2000-2002)
American Society of Biochemists and Molecular Biologists
American Association for the Advancement of Science
American Society for Cell Biology
New York Lipid Club
American Society for Clinical Investigation
Interurban Clinical Club
American Association of Physicians

VII. **Academic appointments**

Assistant Professor of Medicine, Columbia University College of Physicians and Surgeons, New York, NY (1985-1992)
Assistant Professor of Anatomy & Cell Biology, Columbia University College of Physicians and Surgeons, New York, NY (1988-1992)
Associate Professor of Medicine and Anatomy & Cell Biology (**Tenured)**, Columbia University College of Physicians and Surgeons, New York, NY (1992-1997)
Professor of Medicine and Anatomy & Cell Biology (**Tenured)**, Columbia University College of Physicians and Surgeons, New York, NY (1997-present)
Professor of Physiology and Cellular Biophysics (**Tenured**), Columbia University College of Physicians and Surgeons, New York, NY (2004-present)
Vice-Chairman of Research, Department of Medicine, Columbia University (2004-present)

VIII. **Hospital appointments**

Assistant Attending Physician of Medicine, Columbia-Presbyterian Medical Center, New York, NY (1985-1992)
Associate Attending Physician of Medicine, Columbia-Presbyterian Medical Center, New York, NY (1992-present)
Attending Physician of Medicine, Columbia-Presbyterian Medical Center, New York, NY (1997-present)

IX. **Honors**

Phi Beta Kappa, Tufts University, Medford, MA (1974)
Summa cum laude, Tufts University, Medford, MA (1975)
Letter of Commendation, Washington School of medicine, St. Louis, MO (1977)
Mosby Scholarship Book Award, Washington University School of Medicine (1981)
Alpha Omega Alpha, Washington University School of Medicine, St. Louis (1981)
Pfizer Research Award for Young Faculty (1985-1987)]
Silberberg Assistant Professorship of Medicine, Columbia University (1988-1993)
American Heart Association Established Investigator Award (1988-1993)
Doctor Harold and Golden Lamport Research Award (1990)
Elected to the American Society for Clinical Investigation (1992)
Scientific Board of the Stanley J. Sarnoff Endowment for Cardiovascular Science, Inc. (1992-1996)
Editorial Board of *Journal of Biological Chemistry* (1995-2000)
Elected to Interurban Clinical Club (1996-present)
Elected to American Association of Physicians (1998-present)
Deputy Editor, *Journal of Clinical Investigation*, (2002-2007)
American Heart Association/ATVB Council Special Recognition Award (2003)
Named Chair, Department of Medicine (Richard J. Stock Professor of Medicine)
Chairman, 2005 Keystone Symposium on the Cellular Biology of Atherosclerosis
External Advisory Committee, Deuel Research Conferences (2004-2009)
Scientific Board, Kern Lipid Conference (2005-2010)
David Rubinstein Lectureship of the Canadian Lipoprotein Conference (2005)
Closure Lectureship of the 10th Scientific Symposium of the Lilly Foundation entitled "Nutrition, Lipids and Atherosclerosis", El Escorial, Madrid (2006)
Keynote Lecture, Southeastern Lipid Research Conference (2007)
Chairman, 2010 Gordon Conference on Lipoprotein Metabolism

X. **Fellowship and grant support**

<u>Past</u>:

Fellowship: NIH training grant (NHLBI)**,** 1983-85, trainee
Pfizer Research Award for Young Faculty, 1985-1987, Principal Investigator, $50,000 per annum
Project of NIH SCOR Grant in Atherosclerosis (NHLBI), 1986-1990, Co-Investigator, $104,000 per annum
Project of NIH SCOR Grant in Atherosclerosis (NHLBI), 1991, Responsible Investigator, $90,000 per annum
Biomedical Research Support Grant (NIH), 1990-1991, Principal Investigator, $5,000
American Heart Association Established Investigatorship Award, 1988-1993, Principal Investigator, $35,000 per annum
New York Heart Association Grant-in-Aid, Principal Investigator, 1992-1995, $42,000 per annum
Research Supplement for Minority Individuals in Postdoctoral Training (Dr. Anselm K. Okwu)

American Heart Association, New York City Affiliate, Participating Laboratory Award (Dr. Yoshimune Shiratori)
Postdoctoral Fellowship Award in Atherosclerosis (Dr. Paul Skiba)
Schering-Plough Research Grant, 1989-1995, Responsible Investigator, $50,000 per annum
Individual National Research Supplement Award for Postdoctoral Training (Dr. G. Andrew Keesler)
Postdoctoral Fellowship Award in Nutrition (Dr. Sudhir Marathe)
NIH R01 grant (NHLBI), Principal Investigator, 1992-1997, $120,000 per annum
Project of NIH SCOR Grant in Atherosclerosis (NHLBI), Responsible Investigator, 1991-1996, $105,000 per annum
Council for Tobacco Research Award, Principal Investigator, 1995-1998, $75,000 per annum
Postdoctoral Fellowship Award in Atherosclerosis (Dr. Wei Tang)
NIH R01 grant (NHLBI), Principal Investigator, 2001-2004, $215,000 per annum
Research grant from Berlex Laboratories, 2003-2004, $100,000 per annum
AHA Heritage Affiliate Postdoctoral Training Grant, 2004-2005 (Dr. Tracie DeVries)
Merck Sponsored Research Project, Principal Investigator, 2004-2006, $100,000 per annum
NIH SCOR Grant in Vascular Biology (NHLBI), Responsible Investigator or Project and Pathology Core, 2002-2007, $282,000 per annum
AHA Heritage Affiliate Postdoctoral Training Grant, 2005-2007 (Dr. Wahseng Lim)
AHA Scientist Development Grant, 2004-2007 (Dr. Yankun Li)

Present:

NIH P01 grant (NHLBI), Responsible Investigator, 2006-2011, $297,000 per annum
NIH P01 grant (NHLBI), Principle Investigator, 2007-2012, $350,000 per annum
NIH R01 grant (NHLBI), Principal Investigator, 2007-2012, $250,000 per annum
NIH R01 grant (NHLBI), Principal Investigator, 2005-2009, $250,000 per annum
Department of Defense grant, Principle Investigator, 2006-2010, $250,000 per annum
AHA Scientist Development Grant, 2007-2010 (Dr. Tracie Seimon)
NIH Postdoctoral Fellowship Award in Atherosclerosis (Dr. Jenelle Timmins)
AHA Heritage Affiliate Postdoctoral Training Grant, 2007-2009 (Dr. Edward Thorp)
Boehringer-Ingelheim Sponsored Research Project, Principal Investigator, 2007, $150,000 per annum
Fulbright Scholarship (Dorien Schrijvers)

XI. Departmental and university committees

Faculty advisor for Columbia University College of Physicians and Surgeons medical students (1986-1994)
Member of the Columbia University Research Advisory Committee for first year medical student summer research projects (1990)
Member of the Department of Medicine Resident Selection Committee (1990-present)
Organizer of the Department of Medicine Young Faculty Research Conference (1990-1992)
Member of Department of Medicine Subcommittee on Research (1991) and Committee for Organizing Departmental Retreat (1995)
Member of Doctoral Program Subcommittee on Nutrition (1991-present)

Co-Director of Basic Research Track of the CPMC Internal Medicine Residency Program (1992-1997),
Scientific Advisory and Executive Committee, Medical Scientist Training (MD-PhD) Program, Columbia University (1993-present)
Member, Curriculum Committee of the College of Physicians & Surgeons (1997-2002)
Co-Associate Director, Medical Scientist Training (MD-PhD) Program, Columbia University (2001-present)
Chairman, Committee on Promotions of the Department of Medicine (1997-2004)
Member of Search Committees for Director of Pathology, St. Luke's Roosevelt Hosp. (1992), Chairperson of the Department of Pharmacology, Columbia University (1994-1995), Chairperson-Division of Cardiology, Columbia University (1999), Director of the Irving Center for Cancer Research (2004), Chairperson-Division of Oncology (2005)
Member, Dean's Scientific Advisory Committee (2007-)

XII. **Teaching experience and responsibilities**

Specific courses:

Medical Student Preceptor (1989, 1991, 1994, 1996), 6 students
Abnormal Human Biology, Atherosclerosis session preceptor (1987-present), 30 students
Cellular Membranes graduate course (Department of Anatomy & Cell Biology), LDL receptor and intracellular cholesterol metabolism sessions (1987-present), 30 students
Pharmacology graduate student course, LDL receptor session (1989-1993), 20 students
Histology medical student course, microcirculation session (1989-1994), 200 students
Advanced pathophysiology course for fourth year medical, atherosclerosis sessions (1990-1996), 40 students
Pathology graduate student course (Molecular Mechanisms of Disease), organizer and lecturer of Atherosclerosis section (1991-present), 15 students
Science Basic to the Practice of Medicine (formerly Biochemistry of Disease) medical student course, Atherosclerosis session (1992-present), 120 students
Pathophysiology course for 2nd-year medical students, Atherosclerosis session (1997-present), 120 students
Molecular and Cellular Biology of Nutrients, Apoptosis section (2001-), 15 students
Molecular and Cellular Cardiology Lecture Series, Transgenic Models section (1998-), 15 fellows
Molecular Pathophysiology of the Cardiovascular System (2007-), 15 students

General teaching activities:

Attending on Internal Medicine ward service (1985-present), 2-3 students and 3 housestaff physicians
Attending on Endocrinology ward service (1987-present), 1-2 students and 1 fellow
Attending in Combined Endocrine/Diabetes, Thyroid, and Lipid Clinics (1987-present), 1-2 students and 1-2 clinical fellows

Ph.D. Thesis sponsor:

Lori Bottalico, Department of Anatomy/Cell Biology, Columbia University (1989-1992)

Scott Schissel, Department of Anatomy/Cell Biology, Columbia University (1993-1997)
Andrew Leventhal, Department of Anatomy/Cell Biology, Columbia University (2000-2004)—Winner of the 2004 Samuel W. Rover and Lewis Rover Award for Scholarship and Outstanding Achievement in Anatomy and Cell Biology

Masters thesis sponsor:

Sungtae Lim, Institute of Human Nutrition, Columbia University (1989)
Woan-Chyng Su, Institute of Human Nutrition, Columbia University (1990)

Ph.D. Advisory/Examination committees:

Deborah A. Lazzarino, Department of Anatomy/Cell Biology, Columbia University (Ph.D. advisory committee and examination, 1987-1990)
Shing-Jong Lin, Department of Physiology, Columbia University (Ph.D. examination, 1989)
Maria Davila-Bloom, Institute of Human Nutrition, Columbia University (Ph.D. examination, 1989)
Fan Yuan, Department of Engineering, The City University of New York (Ph.D. examination, 1990-1993)
Lester S. Johnson, Department of Pathology, Columbia University, Ph.D. thesis committee (1990-1993)
Steven Rumsey, Institute of Human Nutrition, Ph.D. thesis committee (1992-1993)
Thomas E. Phalen, Albert Einstein College of Medicine, Ph.D. thesis defense committee (1993)
Sripriya Chari, Integrated Program in Cellular, Molecular, and Biophysical Studies, Qualifying Examination (1993)
Zhenglun Zhu, Department of Anatomy/Cell Biology, Columbia University (Ph.D. advisory committee and examination, 1991-1993)
Lori Masucci, Institute of Human Nutrition, Ph.D. thesis committee (1993-1996)
Cory Huang, Department of Pathology, Ph.D. thesis committee (1995)
Mingyue Zhou, Institute of Human Nutrition, Ph.D. thesis committee (1995-)
Hong-yuan Yang, Institute of Human Nutrition, Ph.D. thesis committee (1995-)
Donata Paresce, Department of Pathology, Ph.D. thesis committee (1997)
Furcy Paultre, Institute of Human Nutrition, Ph.D. thesis committee (1997-)
Chris William, Integrated Program. Ph.D. qualifying exam (1997)
Nrgo Storey, Department of Biochemistry, Dalhousie University, Ph.D. examination, 1997
Peter Sartipy, Wallenberg Laboratory, University of Gothenburg, Sweden, opponent, 2000
Ying Lui, Institute of Human Nutrition, Ph.D. thesis committee (1999-)
Edward Cha, Department of Microbiology, Ph.D. thesis committee (2000-2005)
Yu Sun, Institute of Human Nutrition, Ph.D. thesis committee (1997-2002)
Dorien Schrijvers, University of Antwerp, Belgium, jury member, 2007
Suzhao Li, Institute of Human Nutrition, Ph.D. thesis committee (2006-)
Caryn Shectman, Institute of Human Nutrition, Ph.D. thesis committee (2007-)
David Crider, Departement of Pathology & Cell Biology, Ph.D. these committee (2007-)

XIII. **Other professional activities**

Reviewer of over 3000 manuscripts for *Journal of Clinical Investigation, Journal of Biological Chemistry, Journal of Lipid Research, Arteriosclerosis, and Biochimica Biophysica Acta* (1985-present)
Ad hoc grant reviewer for National Science Foundation (1989-present)
Grader for research abstracts submitted to the American Heart Association Annual Meeting (1990, 1992-1994, 1998)
Sub-group reviewer for American Heart Association Established Investigator and Clinical Scientist Award grants (1991 & 1992)
Member of American Heart Association grant-in-aid study section (1992-1993)
Member Scientific Board of the Stanley J. Sarnoff Endowment for Cardiovascular Science, Inc. (1992-1996)
Vice-chairman of American Heart Association grant-in-aid study section (1994)
Consultant for Merck, Schering-Plough, Warner-Lambert, Berlex, Eli Lilly, Pfizer, Talaria Biotech, ReddyUS, Amersham/GE, and Bristol-Myers-Squibb, Novartis, Sankyo
Institutional representative for the American Society of Clinical Investigation (1998-2000)
Co-Editor of October 2000 and 2001 issues of Current Opinion in Lipidology
Organizer and Chairman, Keystone Conference on the Cellular Biology of Atherosclerosis (2005)
External Advisory Committee, Deuel Research Conferences (2004-2009)
Scientific Board, Kern Lipid Conference (2005-2010)
General Council and Review Panel for Future Leaders Grant Program, The Leadership Council for Improving Cardiovascular Care (2005-)

XIV. **Publications** (* indicates that Dr. Tabas is a senior author [*i.e.*, post-graduate school] *and* had a major role in the publication)

Original, peer-reviewed articles:

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XV. **Patents and Invention Reports**

U.S. Patent: "Triterpene Derivatives Cholesterol Acyltransferase Inhibitors and Methods of Using Same"; Ira Tabas, Inventor; January 22, 1991; Patent Number 4, 987,151.

U.S. Patent: "Methods for Treating Conditions with Elevated Levels of Zinc Sphingomyelinase", Ira Tabas, Scott Schissel, and Kevin J. Williams, Inventors. November 23, 1999; Patent Numbers 5,989,803 and 6,613,322.

U.S. Patent: "Human Genetic Clone Encoding Human Chondroitin 6-Sulfotransferase", Kevin J. Williams and Ira Tabas, Inventors. October 10, 1997. Patent Number 6,399,358.

Pending U.S. Patent: "Methods for Identifying Compounds Useful For Preventing Acute Clinical Vascular Events In A Subject".

Pending U.S. Patent: "The use of very low-dose amphipathic amines or others inhibitors of the npc1 pathway to induce ABCA1-mediated macrophage cholesterol efflux, reverse cholesterol transport, and regression of atherosclerotic vascular disease".

Patent Application: "Prevention of Acute Cardiovascular Clinical Events Through Adiponectin and Adiponectin Signaling".

Patent Application: "Phagocyte Enhancement Therapy for Atherosclerosis".